Effect of the Reproductive Cycle on Morphology and Activity of the Ovarian Surface Epithelium in Mammals

Salina Yahya Saddick

Ph.D.

The University of Edinburgh

2010

DECLARATION

I hereby declare that this thesis is my own work and effort, conducted under the supervision of Dr. Evelyn Telfer. It is submitted for the degree of Doctor of philosophy at the University of Edinburgh. No part of this research has been submitted in the past or anywhere for any award. Where other sources of information have been used or assistance given during experiments of this thesis they have been acknowledged

Salina Yahya Saddick

ABSTRACT

The layer of cells lining the outer surface of the mammalian ovary, the ovarian surface epithelium (OSE), is a constant feature throughout the dynamic tissue remodeling that occurs throughout the reproductive cycle (follicle growth, ovulation, corpora lutea formation and pregnancy). Abnormal development of these cells is responsible for 90% of all epithelial ovarian cancers in women and epidemiological studies have shown that susceptibility to ovarian cancer is negatively correlated with increasing pregnancy. Little is known about how OSE cells are affected at each stage of the cycle, so the main aim of this study was to determine how the reproductive cycle affected proliferation and degeneration of OSE cells. This study utilised three animal models each with a different type of reproductive cycle: a mono-ovular seasonal breeder (Sheep), a mono-ovular polyoestrous breeder (Cow) and a poly-ovular non human primate (marmoset) to allow comparisons to be made. Comparison of OSE proliferative activity was made in sheep and marmoset at each stage of the cycle including pregnancy and anoestrous. The bovine model was used to investigate apoptotic cell death.

Proliferative activity of somatic cells within the sheep ovary was monitored throughout the reproductive cycle by detection of cell cycle markers PCNA and Ki67 using immunohistochemistry. The pattern of OSE proliferation was correlated with the pattern of follicle development at each stage (sheep and marmoset). During pregnancy cell proliferation was significantly lower in OSE and in granulosa cells, reflecting a suppression of mature follicle development during these stages whereas in cycling animals proliferation was increased. Differences in OSE proliferation were observed in relation to the local underlying tissue environment in both sheep and marmoset. Epithelial cell rupture and regeneration enhanced the hormonal mitogenic action on epithelial cells, which showed highest proliferation over corpora lutea in each animal model.

To test the hypothesis that these changes are mediated by hormones or growth factors ovine OSE cells were cultured and proliferative activity monitored after treatment with several factors: fetal calf serum (FCS), follicular fluid from follicles of varying sizes, corpora lutea extracts, recombinant human IGF-1,

oestradiol and progesterone. IGF alone was demonstrated to have an affect on increasing proliferation of cultured OSE cells. Levels of FSHr and LHr were monitored by quantitative real- time PCR and it was demonstrated that the concentration of gonadotrophin receptors in OSE, increased prior to and after ovulation, at which time the *in vivo* OSE proliferation also peaked.

The *in situ* apoptosis index was determined in bovine tissue using TUNEL throughout the regular cycle, and at mid and late-pregnancy stages. The results showed that pregnancy induced apoptotic activity in OSE cells and up regulated the tumour suppressor gene *p53*. Cultured bovine OSE cells also exhibited an increased level of apoptosis following progesterone treatment. Since p53/*p53* gene expression in OSE over the corpora lutea producing progesterone also increased, this progesterone-mediated apoptosis may be mediated through an up-regulation of p53 synthesis.

The effect of pregnancy and low production of gonadotrophins in the regulation of OSE cell morphology and activity was further investigated in the marmoset monkey (a non-human primate) treated with GnRH antagonist and infused with BrdU to monitor proliferative activity. OSE proliferation was correlated to ovarian events (follicular growth, ovulation and luteinization) and this was suppressed during pregnancy. Inhibition of gonadotrophin secretion by treatment with a GnRH antagonist also markedly inhibited OSE proliferation.

Taken together these studies support the hypothesis that pregnancy and periods of anovulation reduce proliferation of OSE cells and alter the pattern of apoptotic cell death and that this effect is independent of species and reproductive pattern. Suppression of gonadotrophins and other growth factors during pregnancy could enhance p53-mediated apoptosis of damaged and mitogenic cells arising from repeated ovulations. This effect may partly explain why increasing number of pregnancies in woman reduces the chance of epithelial ovarian cancers.

ACKNOWLEDGEMENTS

It is a pleasure and privilege to express my deepest sense of gratitude to my esteemed supervisor Dr. Evelyn Telfer, for suggesting this problem and encouraging me to take up this challenging work. Her valuable suggestions, constant supervision and enlightening discussions of the research work have always helped me to cross the hurdles throughout the course of investigation. I have greatly profited from her tremendous knowledge on ovarian histology and physiology. I am equally indebted to my second supervisor Professor Hamish Fraser, who has been a fountain head of inspiration and encouragement for the entire tenure. He has worked quite hard with me to complete the investigation. My sincere thanks are also due to all the faculty members of the institute for their support in different ways.

Moreover, I also thank my colleagues and lab mates, in particular Dr. Marie McLaughlin and Mr. John Binnie for helping me under various capacities to execute the work and for their goodwill and cooperation otherwise. My sincere appreciations are also due to Dr Darren Shaw, who helped me with statistical analysis. Special thanks are owed to my husband Khalid Ahmed, who tirelessly supported me morally and academically and never let me feel that I am alone. My Dad, Yahya and Mum Dalal deserve my utmost appreciation for their kind blessings and encouragement that enlightened and instilled in me the belief that I can do. Last but not least my heart goes out for my three kids, Ghaliah, Yazeed and Dalal, their smiles and hugs spirited me to dare into the hard research ventures.

The financial support from Saudi Arabian Government in the form of overseas scholarship, and an offer of a position in the PhD programme of Edinburgh University, deserve special mention and thankful acknowledgement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALK	Activin receptor like kinase
AMH	Anti-Mullerian hormone
AMHR	Anti-Mullerian hormone receptor
ANG	Angiotensin
ANOVA	Analysis of variance
ATF-1	Activating transcriptional factor-1
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BRCA	Breast cancer antigen
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CA125	Cancer antigen 125
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CKs	Cytokeratins
CL	Corpus luteum
CREB	cAMP response element binding protein
DAB	Diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
E_2	Oestradiol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer
EPAC	Exchange protein directly activated by cAMP
Erk	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor

FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
G1	Gap 1
G2	Gap 2
GAGs	Glycosaminoglycans
GCs	Granulosa cells
GDF	Growth and differentiation factor
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotrophin
HGF	Hepatocyte growth factor
HNPCC	Hereditary non-polyposis colon cancer
IC	Inclusion cyst
ICC	Immunocytochemistry
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
IOI	Inter-ovulatory interval
KGF	Keratinocyte growth factor
KL	Kit-ligand
LH	Luteinizing hormone
LHR	LH receptor
LHRH	Luteinizing hormone–releasing hormone
MCM2	Minichromosome maintenance protein-2
MET	Mesenchymal to epithelial transition
$MgCl_2$	Magnesium Chloride
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
OSE	Ovarian surface epithelium
P13K	1-phosphatidylinositol 3-kinase
PBS	Phosphate buffer saline

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PKA	Protein kinase A
PR	Progesterone receptor
RNA	Ribonucleic acid
ROS	Reactive Oxygen species
RT-PCR	Reverse transcription PCR
SEM	Standard error of mean
TA	Tunica albuginea
TBS	Tris Buffered Saline
TGF	Transforming growth factor
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
tPA	Tissue-type plasminogen activator
TRI	Total RNA isolation
Tris	Trishydroxymethylaminomethane
TUNEL	Terminal Transferase-Mediated dUTP Nick End Labeling
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
ZP	Zona pellucida

CHAPTER ONE

Introduction

1.1 General introduction

The mammalian ovary is surrounded by a complete layer of epithelial cells (Ovarian Surface Epithelium; OSE) which has to adapt to the cyclical changes that occur within the ovary before and after ovulation (Gaytan *et al.* 2005). Abnormal changes in the OSE can result in ovarian cancer which is the fifth most common cancer in women and affects about 6700 women annually in Britain (Beral *et al.* 2007). Despite its clinical relevance there is still little known about the features of OSE cells and how they respond to local ovarian factors and cyclical changes. This thesis is concerned with identifying and characterising features of the OSE in relation to stage of the reproductive cycle, ovarian development and paracrine and endocrine factors. This Introduction will provide background to the origin and characteristics of OSE as well as reviewing current knowledge of normal and abnormal OSE regulation within the context of ovarian development and reproductive cycle.

1.2 Embryonic origin of OSE cells in mammals

The embryonic development of human OSE has been described by Auersperg *et al.* (2001). OSE cells are generated from the mesodermally derived epithelial lining of the intraembryonic coelom. In the embryo this covers the presumptive gonadal ridge and proliferates and differentiates to form the gonadal blastema. Within 10-20 weeks of gestation the flat-to-cuboidal simple epithelium with inconspicuous and fragmentary basement membrane changes to multistratified, papillary epithelium with a well defined basement membrane. The cells exhibit short microvilli and wide intercellular cytoplasmic projections (Katabuchi & Okamura 2003). The OSE has characteristic differences from other epithelia, for example expression of cancer antigen 125 (CA125), a surface glycoprotein of unknown function, in the adult is localized in oviductal, endometrial and endocervical epithelium and some extraovarian epithelia, but not in the OSE (Maines-Bandiera & Auersperg 1997; Jacobs & Bast, Jr. 1989). Therefore, either OSE has never acquired this differentiation marker or it is lost early in development (Kabawat *et al.* 1983). CA125 is, however, expressed in tumorigenic OSE suggesting that the original coelomic characteristic has been retained by OSE but is only expressed

under pathological conditions (Jacobs & Bast, Jr 1989). OSE is believed to be the source of at least some of the ovarian granulosa cells (Byskov *et al.* 1977; Hirshfield 1991). The epithelium invaginates as ovarian cords and penetrates deep in the ovarian cortex where it gives rise to the granulosa cells that will form the primordial follicles, which are subsequently detached from the surface epithelium (Figure 1.1). Besides its role as a progenitor of granulosa cells, the coelomic epithelium also protrudes inwards in the vicinity of the presumptive gonads to give rise to Müllerian (paramesonephric) ducts, which constitute the epithelia of the oviduct, endometrium, and endocervix.

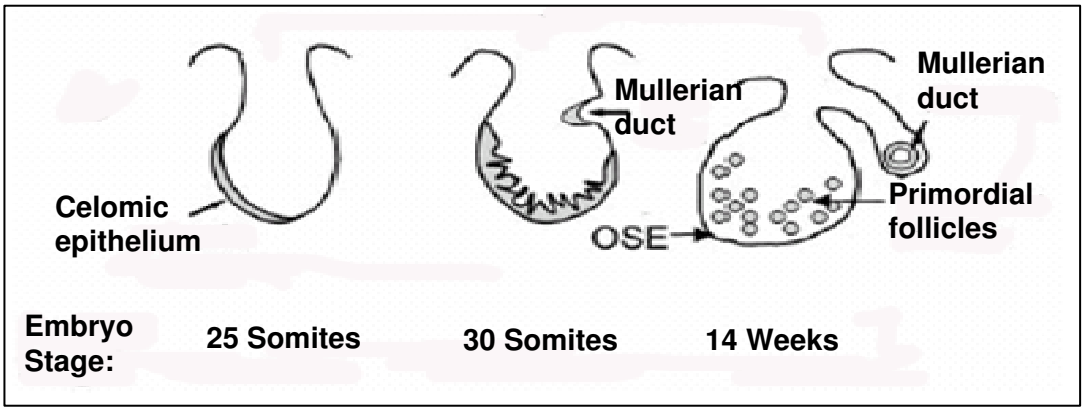


Figure 1.1 Embryonic changes in human gonadal coelomic epithelium which proliferates and forms cords in cortex and gives rise to granulosa cells in primordial follicles that detach from the OSE. The Müllerian duct forms from invaginations of coelomic epithelium. Taken from Auersperg *et al.* (2001).

1.3 Dynamic changes in mammalian OSE structure and function

The OSE is separated from the underlying ovarian structures by a basement membrane and the tunica albuginea. OSE organization in the adult ovary has been described extensively by Auersperg *et al.* (2001). OSE cells have been shown to be held together by zona occludens along their lateral surfaces. Several typical proteins are produced by OSE cells which are different from the other extraovarian epithelia. E-cadherin (epithelial) is a typical calcium-dependent adhesion protein produced in resting surface epithelia of oviduct, endometrium, endocervix and the ovary of mouse and

porcine species (MacCalman *et al.* 1994; Ryan *et al.* 1996) while, it is N-cadherin (neural) that is found in human OSE cells and the granulosa cell lining of the growing follicles (Makrigiannakis *et al.* 1999; Peralta *et al.* 1995). It is believed that when surface epithelial cells undergo transformation into a columnar shape due to metaplastic or neoplastic differentiation, particularly in inclusion cysts and crypts, the E-cadherin starts to co-express along with N-cadherin (Davies *et al.* 1998; Maines-Bandiera & Auersperg 1997). Another form of adhesion protein, P-cadherin (placental) is also typical of Müllerian origin epithelia, but is absent in resting OSE, and occasionally expresses in adenocarcinoma cell lines derived from cancerous OSE cells. Thus, it appears that E-/P-cadherins are induced during OSE neoplasia (Wong *et al.* 1999; van der Linden *et al.* 1994).

The most plausible function of OSE is to transport nutrients, hormones and minerals from the outer peritoneal cavity and to take part in tissue remodelling required for repair at the sites ruptured due to ovulation. OSE also aids follicle rupture and release of the oocyte by secreting lysosomal proteolytic enzymes. It is possible that the OSE secreted substance alters the underlying tunica albuginea and prior to ovulation makes it thin (Bjersing & Cajander 1975). Synthesis of both epithelium (re-epithelization) and connective tissue-like components of extra-cellular matrix may contribute to the rejuvenation of the ovarian cortex. Since connective tissue fibroblasts are responsible for wound healing in diverse tissues and in cultures OSE cells maintain both epithelial and fibroblast mesenchymal characteristics it is reasonable to believe that OSE also regenerates the damaged stroma constituted mostly by mesenchymal cells.

1.4 Inter-relationship between OSE layer and underlying stroma

The epithelial-mesenchymal transition (EMT) is a cellular mechanism that allows the conversion between epithelial and mesenchymal structures. EMT plays an important role during embryogenesis (Hay 1995), it has a capacity towards wound healing in diverse tissue types (Lee *et al.* 2006), and there exists a signalling mechanism for such transition. Normally epithelium to mesenchymal transition requires alterations in morphology, cell architecture, adhesion and mobility. It has been shown that EMT of OSE cells may be a mechanism to repair the perturbed stromal components and also tunica albuginea following every round of ovulation (Ahmed *et al.* 2006). Besides, OSE also helps in weakening the follicular wall through degradation of tunica albuginea and the underlying theca. In sheep and frogs, scraping off the OSE layer hinders ovulation. Hence, this layer facilitates ovulation and post-ovulatory repair. Murdoch & McDonnel (2002) put forth a model based on observations in oestrus ewes, human, mice and pig that explains how OSE interacts with the connective tissue and follicular wall. As shown in Figure 1.2, the most notable actions are collagen breakdown and cellular death at the apex of the preovulatory follicle through apoptosis and inflammatory necrosis. Gonadotrophins circulating in blood of capillaries and venules towards the theca interna stimulate secretion of urokinase-type plasminogen activators (u-PA) from within the OSE cells directed towards the tunica albuginea. Since OSE cell surface luteinizing hormone receptors (LHR) are up-regulated at post-oestrus due to oestradiol produced from preovulatory follicles, it is obvious that gonadotrophin-effect on u-PA synthesis and secretion would be under follicle's control. Besides, coinciding with this time there is also the preovulatory LH surge in higher mammals. Localized accumulation of u-PA triggers conversion of zymogenic plasminogen to a serine protease, plasmin. Unlike tissue-type plasminogen activator (t-PA), u-PA has no role in blood fibrinolysis. Plasmin synthesis occurs in the apical hemisphere of the follicle and conjoined tunica albuginea. Plasmin in turn activates local collagenases and releases tumor necrosis factor-α (TNF-α). The cytokine TNF-α is secreted by thecal endothelial cells of the preovulatory follicle. TNF-α binds to almost any cell, and its action is mediated through activation of TNF-α receptor-R1 (TNFR1) which is constitutively present on the cell surface. Upon

binding to the target cells it can evoke a cascade of proteolytic response which leads to apoptotic DNA fragmentation and cellular disintegration. TNF-α can also activate the transcription of zinc-containing MMP-1 and MMP-2 collagenases. MMP's are synthesized by fibroblast cells in stroma and the steroidogenic granolosa cells of follicles. All these factors collectively degrade the coelomic epithelial layer and follicular granulosal wall at the junction to facilitate ovulation. Basement membranes on which OSE cells and the follicular granulosa cells rest are composed of type IV collagen (Lind *et al.* 2006). The follicular wall is supported by collagen I. Mammalian collagenases belong to group of matrix metalloproteinases (MMP) which degrade collagen. Accumulation of plasmin directly correlates with collagenase activity and with collagen degradation at follicular wall and basement membranes.

After the gondotropin-induced u-PA release from OSE, there is increased activity of apoptosis (plasma membrane phosphatidyl-serine translocation, internucleosomal DNA fragmentation) in the region of the surface epithelium in proximity to the follicle, adjacent tunica albuginea and apical follicle wall. Apoptosis is followed by cellular necrosis, extravasation of blood cells and vascular tissue degeneration (Murdoch *et al.* 1999).

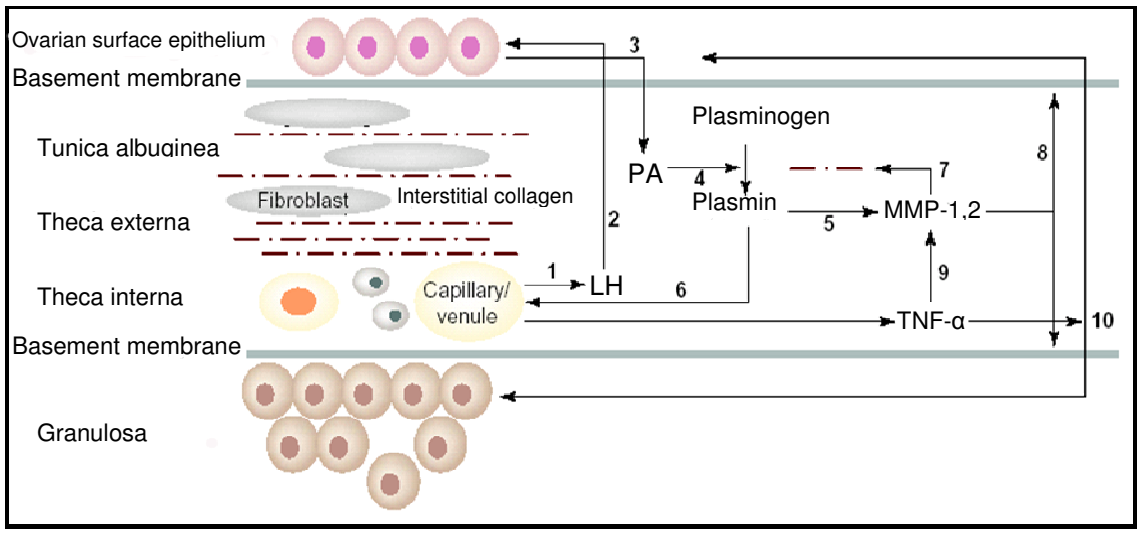


Figure 1.2 Ovulation-related biochemical interaction of OSE and underlying tissues. Thecal vascular LH (1) delivered to OSE (2) stimulates secretion of u-PA (3) that converts interstitial plasminogen to plasmin (4), activates collagenase MMP's (5) and stimulates TNF-α release (6). Collagenases disrupt theca and tunica albugenia (7) and promote digestion of the basement membrane (8) and granulosa cells via MMP, which eventually ruptures the follicle apex (10). Taken from Murdoch & McDonnel (2002).

1.5 OSE and ovarian cancer

The cell of origin of epithelial ovarian cancers (EOC) has been the subject of much debate (Dubeau 2008) but it is thought to originate from the sequestered and detached cells of the epithelial cells lined in the inclusion cysts in the ovarian cortex rather than in the surface monolayer. In the reproductive cycle, and post-menstrual phase the inclusion cysts are formed in typical regular round to oval shapes following ovulation or from mechanical displacement of OSE. These are eventually non-pathologically destroyed with practically no remains left in the ovary. In patients suffering from different stages of ovarian cancer, irregularly shaped inclusion cysts that penetrate deeper into the cortex, become prominently visible besides the regular ones. The irregular cysts are normally not destroyed and continue to proliferate until occasionally they turn malignant. While both types of inclusion cysts have progenitor

cells for cancerous growth, the fundamental difference lies at the level of an ability to self-destroy the epithelial cells in some but not all inclusion cysts. As shown in Figure 1.3, the most common OSE cells observed in ovarian surface, clefts and regularly shaped inclusion cysts are flat to cuboidal, while within irregularly shaped inclusion cysts and tumours the cells that line the tissues exhibit non-ciliated columnar to Müllerian type morphology. The nuclear proliferative marker Ki-67 clearly illustrates the extent of multiplication of different cell types during ovarian cyst development. The flat to cuboidal cells of clefts and regularly shaped cysts do not display any proliferative activity, but a sizable population of columnar cells in irregularly shaped cysts displays extensive proliferation. Consequently, genomic and epigenetic changes initiate pre-neoplasia within such cells, and eventually they lead to advanced cancerous growth.

Unlike in humans, ovarian cyst formation and EOC are extremely rare phenomena in rodents even after artificial gonadotrophin induced super-ovulation. One of the explanations for this is that an anti-proliferative signalling system in OSE prevents detachment of the cells to the underlying stroma after every cycle of normal or artificially induced ovulation. In mice OSE, one of the transforming growth factor-beta family (TGFβ) of proteins; activin, initiates a signalling pathway mediated through phosphorylation of the downstream proteins, SMAD2 and SMAD3 that regulate transcription of a number of genes preventing cyst formation, OSE proliferation and invagination (Burdette *et al.* 2007).

A study was undertaken to ascertain to what extent apoptosis leads to degenerate regular and irregular inclusion cysts, an attribute that determines initial stage of tumourigenesis (Slot *et al.* 2006). It was revealed that expression of apoptosis markers - Fas, Fas ligand, and inactive form of pro-caspase 3 was missing or was redundant in flat to cuboidal cells but started to be seen in the columnar cells of irregularly shaped cysts, borderline tumours and carcinomas. Caspase 3 activation is essential for the ultimate execution of apoptosis signals manifest by surface Fas/Fas ligand (Krammer 1999). There was negligible to low expression of this protein relative to proliferative and other apoptotic markers in the irregular cysts, borderline tumours and advanced carcinomas. A limited number of apoptotic cells among the highly prevalent proliferating cells may lead to a disturbed balance between cell growth and death in irregularly shaped cysts,

and this could be a factor behind the onset of tumourigenesis. Inflammatory response in the cortex releases cytokines - interleukin-1ß, TNFα, IFNα etc. that activate the Fas/Fas ligand mediated apoptosis signalling in all kinds of inclusion cysts (Quirk *et al.* 1997). While in the regular cysts apoptosis leads to degeneration of proliferating cells, in irregular cysts signalling stops beyond pre-caspase 3, thereby initiating the apoptotic pathway but never completing it. As a result, a population of the epithelial cells profusely proliferates but is not subsequently destroyed, and this attribute triggers signals of pre-neoplasia in these cysts (Slot *et al.* 2006).

Mesodermally derived OSE is ubiquitous, expressing both epithelial (keratins) and mesenchymal (vimentin, N-cadherin) markers, but little or no E-cadherin. Clefts and cortical inclusion cysts also rarely express E-cadherin. On the other hand EOC, originating from deep irregular inclusion cysts, clefts, papillary cortical cysts and early tumours express E-cadherin at the initial stage of tumourigenesis. Recent findings (Hudson *et al.* 2008) suggest that unlike the other epithelia in which tumour progression is mediated through EMT, OSE undergoes mesenchymal to epithelial transition (MET) to develop the early tumours.

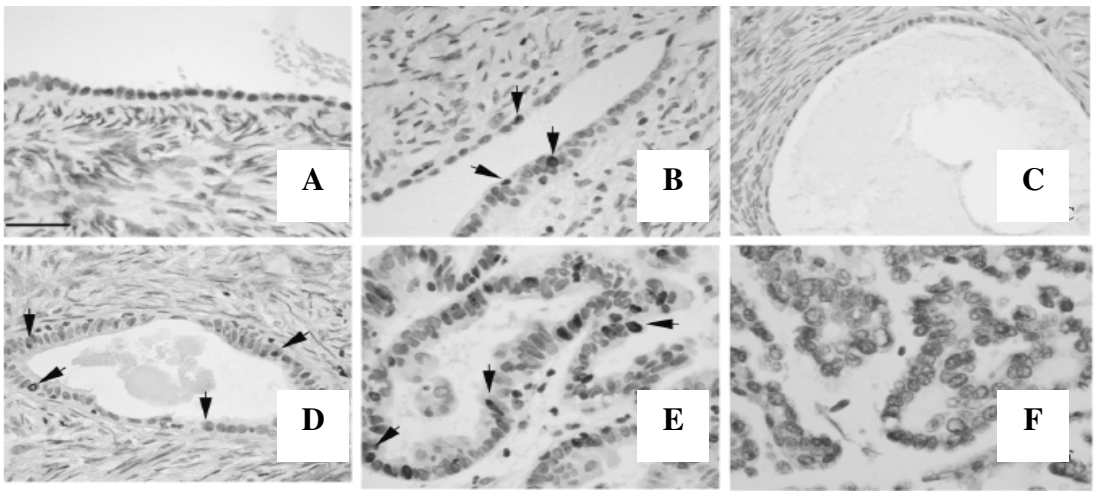


Figure 1.3 Ki67 immunohistology of human ovarian surface (A), clefts (B), regular (C) and irregular (D) shaped inclusion cysts and mucinous carcinoma (E), arrows show positive immunostaining for Ki67 within the nuclei of OSE cells and (F) is the negative control. Taken from Slot *et al.* (2006).

1.6 Histopathological evidence for origin of EOC

It is generally believed that EOC arises from the OSE that covers the ovarian surface (Salazar *et al*. 1996; Feeley & Wells 2001; Connolly *et al*. 2003; Lee *et al*. 2007). However, the cell of origin of ovarian epithelial tumours remains a debatable issue. Auersperg and colleagues (2001) proposed that surface epithelial carcinoma can not be considered in isolation from the other ovarian epithelia derived from Müllerian duct during embryonic development. Histologically, the OSE malignancy can be considered to be serous (oviduct/fallopian tube-like), endometrioid (endometrium-type) and mucinous (endocervical-like) adenocarcinomas, with serous adenocarcinoma comprising about 80% of the total epithelial ovarian carcinoma (Levanon *et al*. 2008). According to Auersperg *et al.* (2008) high grade serous ovarian carcinoma of OSE do not arise from the OSE layer, as was previously thought, but it originates from a distal fimbriated part of the fallopian tube/oviduct, and are only secondarily manifested in metastasis within the ovarian surface. This concept is based on the common embryonic origin of OSE and the Müllerian ducts in the foetal coelomic epithelium. Cheng *et al*. (2005) indicated that the epithelial cells of serous, endometrioid, and mucinous ovarian carcinoma, as opposed to coelomic epithelium, express a set of HOX genes suggesting their common Müllerian origin. Several studies with convincing evidence suggested that the majority of EOC (borderline malignancy and low-grade carcinomas) originate from cortical inclusion cysts (CIC) within the ovarian stroma (Drapkin & Hecht 2002; 2006; Fleming *et al*. 2006). These CIC are lined with epithelial cells resembling those cells lining the Müllerian epithelium that closely resembles the fallopian tube (Levanon *et al*. 2008).

Another school of thought put forward the hypothesis of extra-Müllerian origin of EOC. Secondary Müllerian system are microscopic structures sometimes extending to the ovarian medulla or even in the deeper cortical regions, these structures are lined by Müllerian epithelial cells (Rutgers & Scully 1988).

1.7 Cancer stem cells constitute a small proportion of OSE stem cells

The cyclic and repeated disruption and repair of the OSE with complex remodelling has led to a belief that there exists a population of somatic stem/progenitor cells within the OSE layer responsible for sustained wound healing (Szotek *et al*. 2008). Somatic stem cells are just the normal tissue cells with an ability to renew themselves by asymmetric division, and thereby they produce a set of daughter cells committed to rectify the damage by regeneration and repair. Parrot and colleagues (2000) investigated the expression of markers specific to somatic stem cells in the human and bovine OSE layer. Both human and bovine OSE cells exhibited positive staining for Kit ligand (KL) and its tyrosine kinase receptor *c-kit* which are typical stem cell factors (Parrot *et al.* 2000a).

Increasing evidence supports the hypothesis that OSE tumour growth capacity depends on cancer stem cells (CSC's) that arise from a small proportion of OSE stem cells (Pan & Huang 2008). It has been reported that CSC's are responsible for aggressiveness of the disease, metastasis and resistance to chemotherapy (Bapat *et al*. 2005). In EOC cell, a small subset of tumour stem cells, called side population (SP), actually proliferates as CSC's and the rest of the population behaves like stem cells destined for damaged tissue repair. SP can be detected by their ability to efflux the DNA-binding dye Hoechst 33342 through an ABC membrane transporter (Pan & Huang 2008). In a mouse model, several proliferative markers were used to distinguish the quiescent population of OSE cells from highly proliferative cells surrounding the post-follicular wounded region in OSE layer (Szotek *et al.* 2006). A significant result was that two nuclear proliferation markers, bromo-dUracil (BrdU) and histone 2B green fluorescent protein (2HB-GFP) were retained by a quiescent cell population for up to four months, while another population within the same tissue rapidly lost the markers in a short period (Szotek *et al.* 2006). With high resolution confocal microscopy these authors demonstrated the possibility of asymmetric division of coelomic epithelial cells, which is highly characteristic of stem cells. The conclusion was that those cells which lost the markers were highly proliferative somatic stem cells showing asymmetric

division, and were distinct from the OSE tissue specific cells that were dividing less and as a result maintained the nuclear markers for longer periods (Szotek *et al.* 2006).

1.8 Epidemiology and heredity of epithelial ovarian cancer (EOC)

Ovarian cancer is the fifth leading cause of cancer deaths, and results in the highest mortality rate among female cancers (Jackson *et al.* 2009). Over 90% of ovarian cancers arise from surface epithelium (Murdoch & McDonnel 2002). The extent of lethality in this cancer is the highest among various malignancies. At the initial stage the five year survival chances are only 45% and as the disease advances, the chances in the patients reduce further to 30% (Ponnusamy & Batra 2008; Landen, Jr. *et al.* 2008). Most ovarian cancers have no racial or ethnic prevalence, even though the incidences are generally higher in the Western countries. Though occurrence of ovarian cancer is recognized as sporadic, about 5-10% of incidences have familial history and risk among first-degree relatives (mother, sister and daughter) increases by 50% (Murdoch & McDonnel 2002). Wong & Auersperg (2003) and Sowter & Ashworth (2005) reviewed the nature of inheritable traits responsible for familial lineage of EOC. In many cases people carrying germline mutations in one of the alleles of the tumour suppressor genes *BRCA1* (Breast cancer antigen 1) or *BRCA2* are at significantly higher risk of acquiring breast or ovarian cancers. The *BRCA1* and *BRCA2* genes code for proteins that are responsible for DNA double stranded breaks by homologous recombination (Tutt & Ashworth 2002). Consequently, several chromosomal abnormalities and genetic instability lead to onset of cancerous transformation of mammary and ovarian epithelia. While BRCA protein is widely expressed in all kinds of cells, the prevalence of *BRCA* mutations in some tissues and not others, relates to the microenvironment of a particular tissue which becomes crucial for pathogenesis. Women inheriting mutations of *BRCA1* or *BRCA2* genes have a ~40% or ~10% risk, respectively, of developing ovarian cancer by the age of 70. To a lesser extent, ovarian cancer risk increases in families with cases of heredity non-polyposis colon cancer (HNPCC). Internalization of OSE to form inclusion cysts is a highly proliferative act of the epithelial cells, which happens cyclically in the ovary at ovulation (Sowter & Ashworth 2005). Despite the

genotoxic/mitogenic effect of the local environment and inhibition of apoptosis, epithelial cells can maintain healthy progression so long as the BRCA proteins remain functional. Figure 1.4 shows the process of follicle ovulation and subsequent events leading to EOC.

Epidemiological studies revealed that the number of ovulations plays an important role in the initiation of ovarian cancer (Fathalla 1971; Meisler 2000), and repeated cycles of ovulation increases the risk of ovarian cancer (Tiedemann 2000). Contrary to this, factors that suppress ovulation process such as pregnancy, administration of oral contraceptives and lactation have been shown to exert a protective role against onset of ovarian cancer (Tiedemann 2000). Epidemiologic and cohort studies point to possible association between superovulation drugs and increased risk of ovarian cancer (Whittemore *et al.* 1992b; Rossing *et al.* 1994). However, a subsequent study supported by international pooled analysis of large sample showed no overall elevated risk of ovarian cancer after use of fertility drugs and gonadotrophins (Ness *et al.* 2002; Jensen *et al.* 2009).

Normally, glucocorticoids, like oestradiol, are believed to act as mitogenic and inflammatory agents enhancing the OSE cell proliferation at post-ovulatory damaged sites and may induce neoplastic transformations unless these cells are periodically removed by apoptosis. However, in a recent study using cultured human OSE cells it was found that there exists a local intracrine mechanism operational under inflammatory responses like interleukin stimulation, that enable OSE cells to overcome the damage during ovulation, A number of cytokines are known to be released and are present in high concentrations during the ovulation process (Kenny *et al.* 2008). Rae *et al.* (2009; 2004) have worked on this system and proposed for the first time that cortisol, synthesized by the enzymatic activity of type I 11β-hydroxysteroid dehydrogenase, inhibits the expression of several inflammation-related genes, and thereby act as an anti-inflammatory agent. This anti-inflammatory cortisol in conjunction with paracrine progesterone suppresses the expression of matrix metalloproteinase (gelatinase), which is otherwise up-regulated in response to diverse ovulation-associated inflammatory agents, like cytokines.

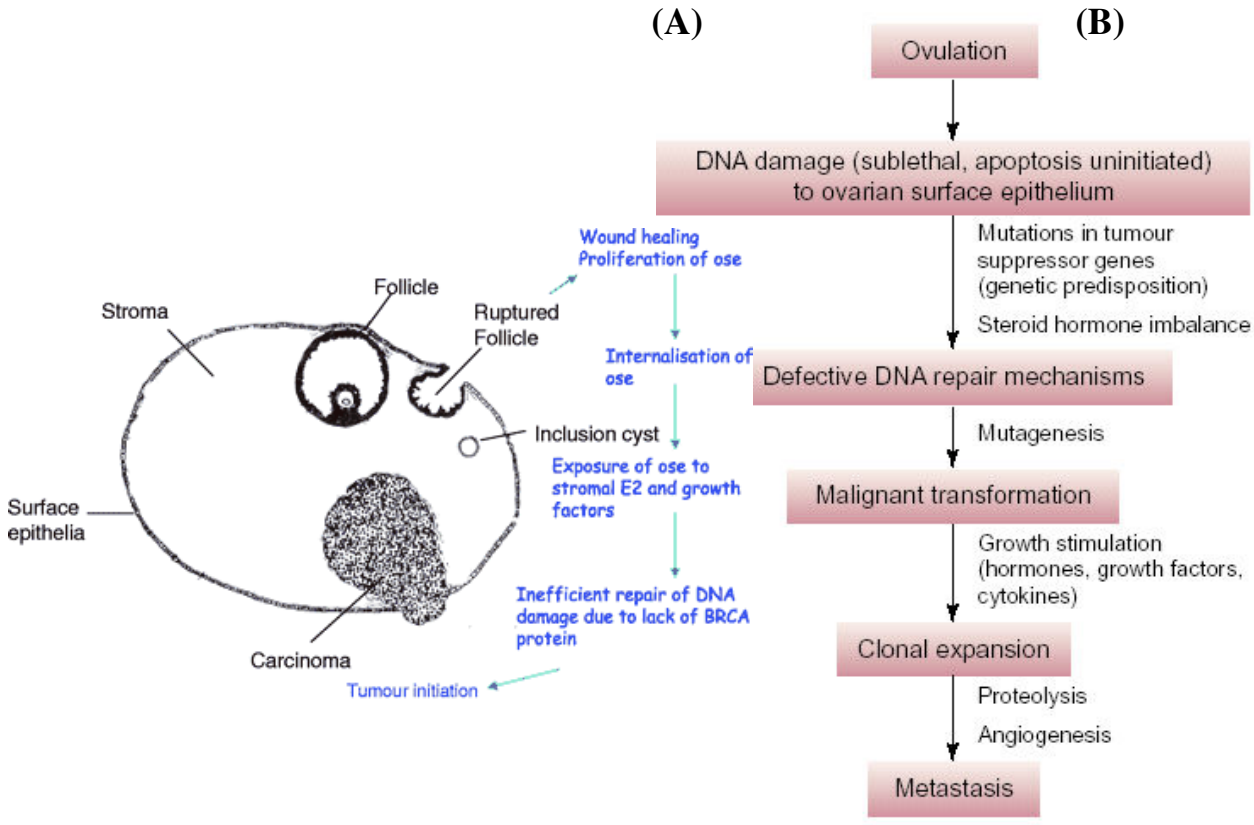


Figure 1.4 Diagrammatic representations of the normal ovulatory cycle and events leading to EOC. (A) Progressive role of ovulation in chronology of EOC and (B) OSE during repeated ovulation and mitogen exposure and accumulated mutations particularly in individuals with *BRCA* lesions invaginates as inclusion cysts and epithelial cells turn malignant and then manifest as serous carcinoma. Adapted from Murdoch & McDonnel (2002) and Sowter & Ashworth (2005).

1.9 Ovarian follicle development

The ovary is a dynamically changing environment and the OSE cells are likely to interact with the underlying stroma and follicles at all stages. In mammals, OSE assists in dissolution of the follicle apex and all the underlying connective tissue and helps in releasing the matured ovum. Further, the mitogens mostly present in follicular fluid influence the OSE proliferation, re-epithelization and tumourigenesis. Given this nature

of intimate relationship between the two integral tissues of ovary, it is necessary to discuss in the following section the key features of mammalian follicle development.

The oocytes and their surrounding somatic cells constitute the fundamental reproductive unit of the ovary, the ovarian follicle. The ovarian cortex of a young woman is largely filled with progressively developing follicles, whereas with aging due to repeated ovulation and atresia most of the follicles are replaced by fibrous tissue. The maximum number of ovarian follicles is found just prior to birth, and this gradually declines during childhood and then drops sharply before menopause leaving just ~1000 aging follicles which are non-ovulatory (Faddy & Gosden 1996). There are two distinct yet complementary processes that contribute to follicle development; oogenesis and folliculogenesis.

1.9.1 Oogenesis

The germ cells originating from embryonic ectoderm migrate during early gestation to the genital ridge. In humans, once the gonad has differentiated as an ovary, germ cells develop into oogonia (between 7 and 9 weeks gestation). These oogonia continue to multiply through mitosis (Faddy *et al.* 1992) until they start to enter meiosis (around 10-11 weeks gestation) and oocytes become arrested at diplotene stage of the first meiotic prophase. These oocytes are termed primordial oocytes. By the fifth month of human gestation about 7 million oocytes are produced, followed by extensive wave of atresia (Baker 1963) of which only 2 million remain at birth. At puberty approximately 400,000 primary oocytes still remain (Faddy 2000), and this is the quota for the reproductive phase of a woman. From this population of oocytes only a maximum of 400-500 (0.1%) of follicles mature and ovulate, whereas the remainder become atretic.

Recent studies have suggested that postnatal oogenesis may also occur in female mammals (Johnson *et al.* 2004). They suggested that germline stem cells can repopulate the postnatal ovary and renew the primordial follicle pool. This group subsequently went on to suggest that these cells were derived from bone marrow (Johnson *et al.* 2005). This has attracted a great deal of attention as well as criticism (Gosden 2004; Byskov *et al.* 2005; Telfer *et al.* 2005). This is an ongoing debate but the balance of evidence suggests that renewal is not a major factor in ovarian development (Eggan *et al.* 2006).

From the onset of puberty to menopause approximately 15-20 primary oocytes are released into the growing pool in each menstrual cycle, completing the meiosis-I and continuing through meiosis-II until metaphase. The second meiotic cycle only completes upon fertilization.

1.9.2 Folliculogenesis

This is a sequential process in which the follicles mature from primordial to the preovulatory stage. The pool of primordial follicles develops during fetal life in some species (e.g primates, ruminants), but in others it develops during the early neonatal period (e.g rodents, rabbits) (Marion *et al.* 1971). Two major processes accomplish this development, a) recruitment of the follicle in to the growing follicle pool, and b) proliferation and differentiation of theca and granuolsa cells. The first process is controlled by localized autocrine and paracrine factors produced within the ovary, whereas the latter is regulated by both localized internal factors as well as the endocrine signals from outside the ovary.

1.10 Stages of follicular development

The quiescent primordial follicle in bovine (diameter, ~40 µm) is constituted by the immature oocyte surrounded by a single layer of squamous (flattened) pre-granulosa cells, and covered by a basement membrane which separates the follicles from surrounding ovarian cortex (Gosden 2002). Two events transform primordial follicles to primary follicles, a) granulosa cells start to proliferate and grow into large cuboidal cells, and b) transcription in the oocyte is activated resulting in cell-to-cell paracrine signalling between the oocyte and surrounding granulosa. At this stage both the cell types start to grow and mutually interact. Primary follicles (diameter, 40-80 µm; bovine) are the first level of activation of the recruited primordial follicles. A significant property of granulosa cells is that they secrete mucopolysaccharides around the growing oocytes to form the zona pellucida (ZP) (Epifano *et al.* 1995; Wassarman 1988). ZP is a thick layer of glycoproteins and proteoglycans between oocyte and the granolosa layer. The exact

source of ZP glycoproteins is not clearly established because both granolosa and primordial oocyte have been shown to produce these proteins (Gook *et al.* 2008).

Occasionally, a fraction of the pre-granulosa cells transform to cuboidal granulosa cells typical of primary follicles. Surrounding the basement membrane are the cumulus cells that keeps a gap junction with the oocyte plasma membrane by projecting their microvilli through the ZP (Li *et al.* 1995). This allows bidirectional exchange of nutrients, metabolite precursors, and signal molecules, including growth factors (Eppig 1991). As granulosa cells proliferate further and form multiple layers around larger oocytes, the follicles differentiate into secondary or preantral follicles (diameter, 80-250 µm; bovine). Additional stromal cells from the surrounding region are recruited over the basement membrane to produce two distinct layers, called theca interna and theca externa. At this stage the follicles start to obtain their blood supply through microvascular tissue developed between the two theca layers (Reynolds *et al.* 1992). With the advent of blood supply, circulating follicle stimulating hormone (FSH) facilitates formation of a multi-lamellate follicle of many layers of actively dividing granulosa cells. FSH receptors begin to appear on the granulosa cell surface, while LH receptors are exclusively seen in theca cells.

The early antral follicle (diameter, 250-500 µm; bovine) contains a fluid filled sac called the antrum, which contains blood exudates and local secretions including hormones and metabolites. Upon stimulation with LH the theca cells produce androgen, which is then converted to oestrogen within the granulosa cells. A large fluid-filled Graafian or antral (tertiary) follicle represents a mature follicle. These follicles contain two distinct layers of granulosa cells - the cumulus granulosa cells surrounding the oocyte and mural granulosa cells along the follicle wall. Bovine preovulatory follicles may reach a size of approximately 20 mm. During the ovulatory cycle many antral follicles start to grow but only one will ovulate with the rest becoming atretic. Figure 1.5 presents an outline of different stages of folliculogenesis.

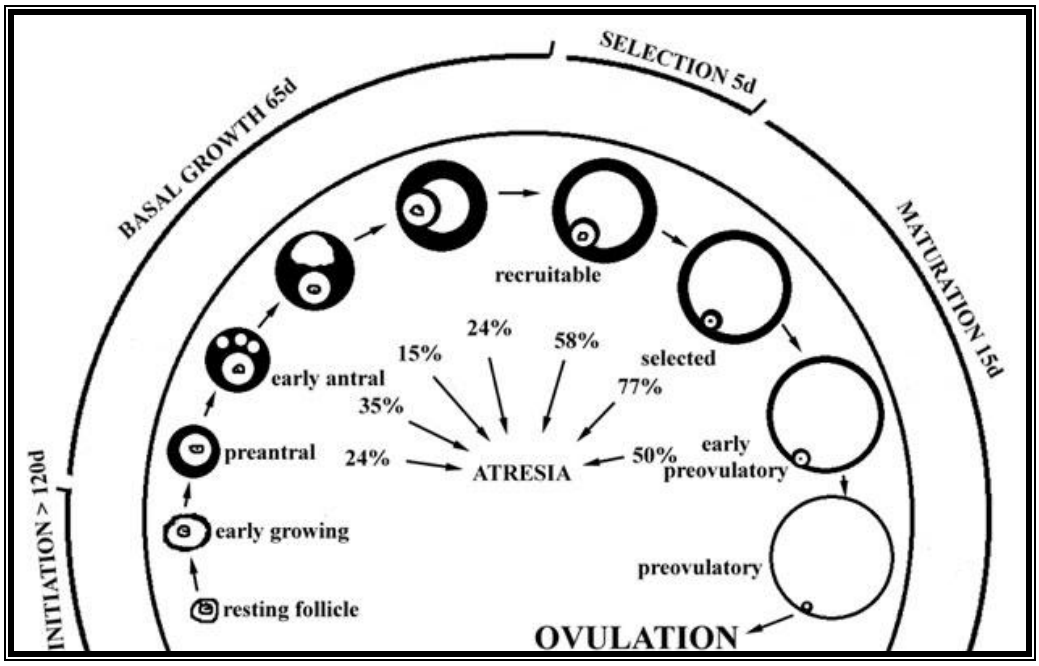


Figure 1.5 Folliculogenesis and classification of developing follicles in human ovary. Taken from Gougeon (1996).

1.11 Follicular growth during the menstrual and oestrus cycle

Analysis of development of large follicles (secondary to preantral) in mammalian species shows that their development occurs only at particular reproductive states or during particular times of the reproductive cycle (Fortune 1994). In humans and other primates, a large ovulatory follicle only appears at a particular time rather than randomly during the cycle. A cohort of growing follicles emerges at the early follicular phase and only one follicle continues to grow through to the late follicular phase whereas the rest of the cohort regresses. No ovulatory size follicles develop in the luteal phase. In the other species (cattle, horses, sheep) follicular development involves the development of ovulatory size follicle throughout the cycle, including the luteal phase. Two-to-three times during the cycle a "wave" of 3-6 follicles continues to grow until they reach a size close to 5 mm. One of them, slightly larger than the others, continues to grow further while the remaining subordinate follicles regress. Follicular waves also occur during the luteal phase and even during pregnancy and the pre-pubertal phase. The emergence of

each follicular wave in ewes (Toosi *et al*. 2010) and heifers (Adams *et al*. 1992) is preceded by a sharp increase in circulating FSH concentrations.

Conventionally, the ovarian cycle in women starts on the first day of menstruation, it commences with a follicular phase followed by "mid point" ovulation and then a luteal phase. In between the two consecutive cycles there exists an inter-ovulatory interval (IOI) which starts with luteal phase of the preceding cycle followed by the follicular phase of the next cycle. It has been recently demonstrated by Baerwald *et al.* (2003) that selection of a dominant follicle over the subordinate follicles in women occurs not just once during the follicular cycle as perceived earlier, but 1-3 times during IOI which is in consistent with the observation with the bovine and equine. Distinct major and minor waves of follicular growth were observed in ovaries of women and mares (Baerwald *et al.* 2003; Ginther 1993). Major waves were those in which dominant follicle was selected for further growth until ovulation, while in minor waves growth and dominance of the follicle was not manifest. In bovine species all waves during IOI were major and only the last one called "ovulatory wave" led to the development of the dominant ovulatory follicle. The dominant follicles in preceding waves did develop but then these underwent regression, resulting in "anovulatory waves". In contrast, women exhibited major and minor waves during IOI. The final wave of IOI was a major ovulatory wave and the preceding waves were either anovulatory major waves or were minor waves. In women too, the circulating FSH surge was associated with anovulatory major waves in IOI, but the ovulatory wave was independent of FSH level. Hence, for all practical purposes the mammalian species exhibit a similar wave pattern of folliculogenesis in the IOI phase.

1.12 Local regulatory growth factors/hormones and signalling molecules

1.12.1 Transforming growth factor-β (TGF-β) superfamily in follicle recruitment and development

About 40 proteins with similar structures including six cysteine residues belong to this superfamily that influences the growth and development of the follicle (Erickson & Shimasaki 2000). Broadly, three categories of growth factors namely TGF-β itself;

activins and inhibins; and bone morphogenetic proteins (BMP's) and growth and differentiation factors (GDF's) induce a cascade of signal pathways in growing granulosa through two transmembrane serine/threonine kinase receptors, the activin receptor like kinase 5 (ALK5; type I) and BMP receptor II (BMPRII; type II). According to Hutt & Albertini (2007), GDF-9 is expressed in oocytes throughout folliculogenesis. Deletion of the *gdf-9* gene in mice resulted in infertility with ovaries in which follicle development was hindered due to lower proliferation of granulosa cells and reduced ability in assembly of theca cells. Eventually the oocytes degenerated. GDF-9 signals are mediated through type I and II receptors and the SMAD2/3 pathway in granulosa cells. BMP-15 is a homologue of GDF-9 and is also constantly secreted by oocytes. Mutation in the *bmp-15* gene arrested follicle development at the primary stage and oocytes were lost from granulosa cell clusters. BMP-15 binds to type II receptor and functions in conjunction with GDF-9. More recent findings supported a hypothesis that these factors contribute to a bidirectional cell-to-cell interaction through gap junctions and paracrine signalling between oocytes and surrounding mural granulosa and cumulus cells in preantral follicles (Li *et al.* 2008). This attribute is advantageous for oocyte competence and good quality, being regulated by the somatic cells. BMP-15 and BMP-6 protect the cumulus cells from undergoing apoptosis, and thereby the oocyte creates a favourable microenvironment of surviving cumulus cells (Hussein *et al.* 2005). GDF-9/BMP-15 cooperatively regulates cholesterol biosynthesis in cumulus cells (Su *et al.* 2008).

During early folliculogenesis, GDF-9/BMP-15 stimulates Kit ligand (KL) expression in granulosa cells. Together with its tyrosine kinase receptor c-Kit present in oocytes, KL is responsible for survival, growth and differentiation of primordial germ cells. KL/c-Kit in a paracrine mode down-regulates the expression of GDF-9/BMP-15 and thereby creates a negative feedback loop between granulosa cells and the oocyte. Hutt *et al.* (2006) elucidated a role of KL/c-Kit in folliculogenesis. Activation of primordial follicles and recruitment is promoted by KL. KL may also be involved in survival of primordial and preantral follicles most likely by evading apoptosis. In the growing follicle, KL also maintains the arrested status of meiosis-I. The LH surge decreases production of KL in cumulus and mural granulosa, a condition that resumes oocyte meiotic division. KL has also been shown to facilitate theca cell recruitment

(Parrott & Skinner 1998) and interaction between theca and granulosa cells in preovulatory follicles, especially the step of oestrogen biosynthesis from androgen (Parrott & Skinner 1997).

According to Nilsson *et al.* (2007), transition of primordial to primary follicle is controlled by inhibitory and stimulatory growth factors. Among the stimulatory factors prominent are fibroblast growth factor-2 (FGF2) and KL (Nilsson *et al.* 2001b; 2006). One of the inhibitory factors is Anti-Müllerian hormone (AMH), a member of TGF-β superfamily which binds to AMH receptor 2 (AMHR2) on the granulosa cell surface and suppresses proliferation by way of up-regulation of genes under the control of SMAD proteins (Carlsson *et al.* 2006). AMH is also reported to partially block the binding of FSH and LH to their receptors and the gonadotrophin-induced follicle development is also consequently suppressed (Durlinger *et al.* 2001). In microarray analysis, AMH was found to negatively interact with different stimulatory growth factors specific to oocyte and granulosa/theca cells, and affect the downstream signalling pathway necessary for follicle transition and development (Nilsson *et al.* 2007).

1.12.2 Angiogenic factors

The second important component of follicle development is the vasculature for blood supply of oxygen, nutrients, hormones and removal of CO_2 arising from respiration. According to Bruno *et al.* (2009) a wide range of pro-angiogenic and anti-angiogenic factors regulate folliculogenesis and any perturbation leads to atretic degeneration of the follicle at any stage of development. Quiescent primordial to slow growing preantral follicles do not have a blood supply of their own, but as the antrum develops the thecal layer acquires vasculature in the form of two capillary networks in theca interna and externa (Stouffer *et al.* 2001). The most crucial granulosa cell-produced angiogenic factor is the Vascular Endothelial Growth Factor (VEGF) (Fraser & Duncan 2009), responsible for establishing and increasing the density of the capillary network and assembling the circulating endothelial cells. The assisting pro-angiogenic growth factors that function in conjunction with VEGF are FGF-2 and angiotensin (ANG II) (Redmer *et al.* 2001). VEGF also enhances capillary permeability and

maintains endothelial cells for microvascularization (Bruno *et al.* 2009). VEGF is produced in oocytes of primordial to primary follicles (Ferrara *et al.* 2003) but in dominant follicles granulosa and theca cells take over this function (Barboni *et al.* 2000). Angiogenic endothelial cells display positive VEGF receptor expression, and they start to assemble towards inner theca as initial steps of vascularization.

1.12.3 Systemic hormones in follicle development

In different mammalian species the pituitary gonadotrophins (FSH and LH) and insulin-like growth factor I and II (IGF-I and IGF–II) have considerable impact on the later development of multi-lamellar follicles and on ovulation (Ryan *et al.* 2008; Campbell *et al.* 2009). In fact, the effect of the gonadotrophins is seen mostly on the dominant ovulatory follicle when the other sub-ordinate follicles start to regress. Both gonadotrophins, FSH and LH induce steroidogenesis and proliferation via binding to specific G protein-coupled receptors, which lead to a rise in cyclic adenosine monophosphate (cAMP) and activation of the protein kinase A (PKA) pathway (Seger *et al.* 2001), which is considered as the principle mediator; additionally these hormones stimulate other signalling pathways; the extracellular signal-regulated kinase (Erk) (Babu *et al.* 2000) and Akt (Zeleznik *et al.* 2003) pathways. Beside gonadotrophins other growth factors, mainly IGF's, stimulate theca and granulosa cell proliferation and augment gonadotrophin action to stimulate steroidogenesis (Davidson *et al.* 2002). In addition IGF's display high anti-apoptotic effect primarily on the dominant follicle, while its expression diminishes in the atretic subordinate follicles that are prone to apoptotic degeneration (Chamoun *et al.* 2002). These signalling pathways up-regulate the production of progesterone and oestradiol in the granulosa cells. In addition, the follicles also express high levels of TGF-β family growth factors like inhibin-A, activin-A and AMH, which interact with gonadotrophin-mediated signal transduction. Thus, local growth factors not only modulate the gonadotrophin-independent primordial to primary follicle development, they also regulate LH and FSH action on granulosa and theca cell proliferation at later stages of folliculogenesis. BMP-6 is a factor that augments the gonadotrophin action by increasing FSH induced oestradiol and inhibin A

production while decreasing FSH induced progesterone production by granulosa cells (Campbell *et al*. 2006; Otsuka *et al*. 2001). AMH acts as an inhibitor of gonadotrophin-induced follicle development without affecting steroidogenesis. Further, BMP-15 and GDF-9 exert an inhibitory effect on LH-induced thecal steroidogenesis. Increased activity of gonadotrophin augmenters (BMP-6) and decreased activity of gonadotrophin attenuators (AMH, BMP-15, and GDF-9) may increase FSH sensitivity resulting in alteration in the mechanisms of follicle selection (Campbell 2009).

1.13 Rationale of endocrine and developmental factors in ovulation

According to Diaz *et al.* (2007), preovulatory antral follicles are comprised of somatic cells that exhibit both endocrine and developmental functions. The cumulus cells in proximity to the oocyte obtain the developmental signals responsible for growth and competence of the oocyte via the micro-environment created by adjoining cumulus cells (Figure 1.6). Prior to the LH surge, oocyte-derived TGF-β signaling pathways regulate the function of cumulus cells and oppose FSH action. Consequently, cumulus specific transcripts, *Slc38a3*, *Amh* and *Ar*, required for oocyte growth and competence are produced in cumulus cells. Opposing FSH action maintains the cumulus phenotype in these granulosa cells without them being converted to mural granulosa cells. FSH interacts with the mural granulosa beneath the follicle wall and induces transcripts for steroidogenesis (*Cyp11a1*), ovulation (*Lhcgr*) and immune function (*Cd34*). After the LH surge, not much change occurs in mural granulosa, but the mRNA expression profile of cumulus cells radically change to cumulus expansion specific transcripts, *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip*, which enables detachment of the developed oocytes in the antral fluid for release upon follicle rupture (Diaz *et al.* 2007).

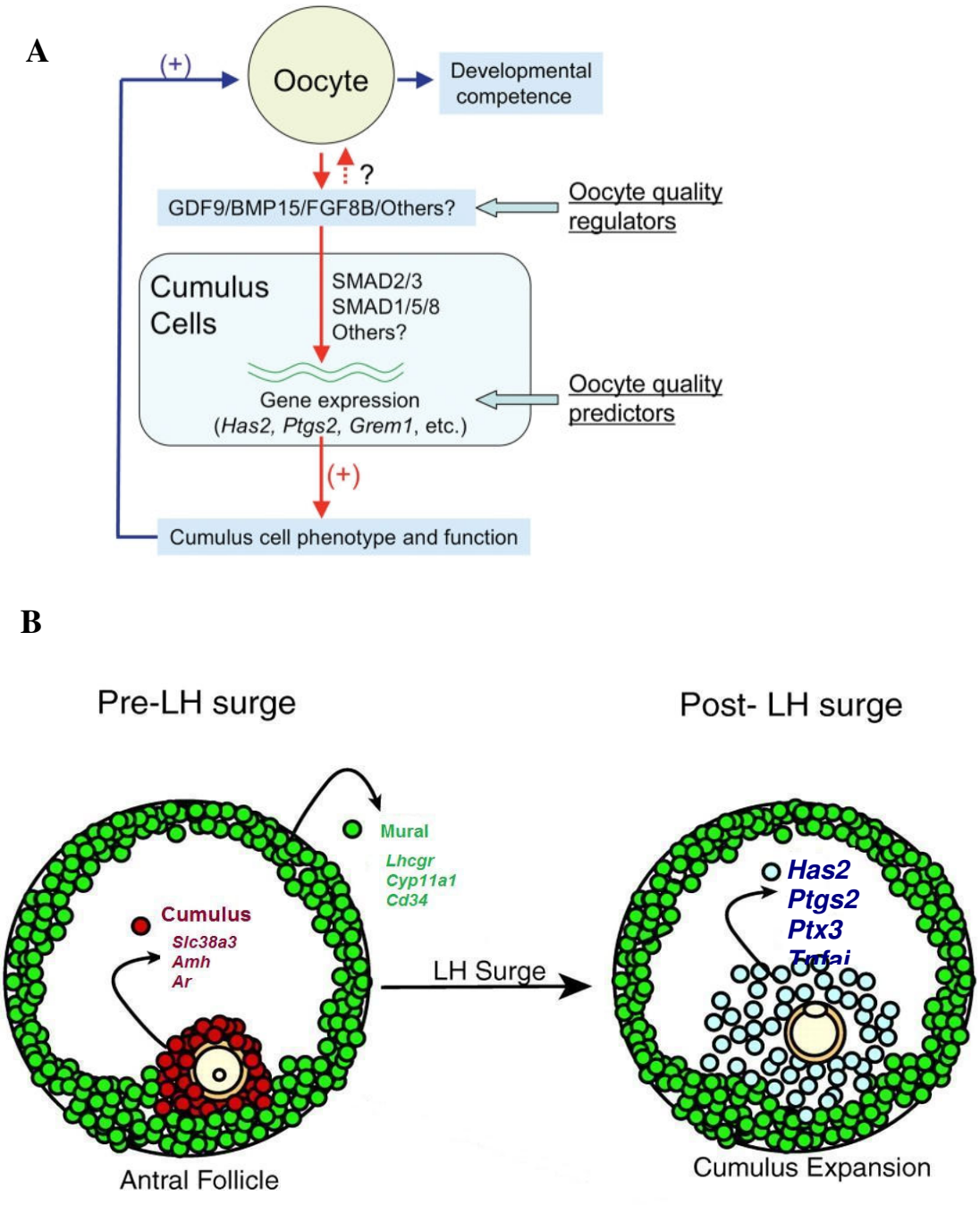


Figure 1.6 (A) Oocyte-cumulus cell regulatory loop showing release of TGF-β factors which influence cumulus phenotype enabling oocyte developmental competence. (B) Shifting of gene expression due to LH surge and expression of cumulus expansion genes enabling freedom of oocyte for release. Adapted from Li *et al.* (2008) and Diaz *et al.* (2007).

1.14 Aims of the project

Epithelial ovarian cancer is thought to arise from ovarian surface epithelial cells that are entrapped within the ovarian cortex during or after ovulation. Epidemiological studies reveal that pregnancy and progesterone-based contraceptive drugs play a protective role against ovarian cancer though the underlying mechanism is largely unknown. Although OSE has attracted the attention of researchers due to its structural and functional involvement in progression of over 90% of the ovarian cancers, a systematic investigation of such a fragile layer has been difficult for several reasons, namely a) OSE comprises a small fraction of the whole ovary, therefore only a small number of cells can be obtained for the *in vitro* study, b) in cultures OSE cells take around 3-4 weeks to attain confluence, and c) it is difficult to compare *in vivo* and *in vitro* conditions. It is therefore essential to study OSE morphology *in vivo* and to determine how underlying ovarian tissue and stage of cycle interacts with OSE cells.

Much of the earlier research on OSE , particularly its involvement in neoplastic development, used the mouse as a model but this is not the best model for human since: a) ovarian senescence and malignancy in cycling and breeding mice is quite rare and so experimental material would not be normally available unless artificially induced, b) although there are some recent contradictions, mouse OSE cells are believed to be held together with E-cadherin whereas for human it is N-cadherin, and this difference has direct implication in mesenchymal-epithelial transformation, c) human epithelial ovarian cancer often forms metastasis in the peritoneal cavity and ascites, which may be fundamentally different in the mouse due to the presence of a bursa sac that forms a physical barrier around the ovary preventing immediate access to the peritoneal space, and d) precancerous surface marker, CA-125 is not produced in mice. Therefore models such as sheep and cows that share many similarities to human may be more appropriate

The work presented in this thesis is the first to carry out an *in situ* morphological and functional analysis of OSE cells at different stages of ovulatory and luteal cycles, and during pregnancy using different animal model systems. This allows us to tackle the question of whether the effect of pregnancy on OSE cells is a general phenomenon seen in other species.

The specific objectives pertaining to each chapter embodied in this thesis are:

(1) To examine the proliferative activity of the ovine OSE cells through the regular ovarian cycle and during pregnancy in ovine model by monitoring proliferation *in vivo* using immunohistochemistry (IHC) of proliferation markers PCNA and Ki-67, and also to investigate whether the underlying growing follicles have any influence on OSE layer. This was achieved by monitoring the follicular dynamics and proliferation during oestrus and anoestrus stages and during pregnancy (Chapter Two).

(2) To examine, using cultured ovine OSE cells, a role of local ovarian components, namely growing follicles and corpora lutea and their chemical constituents - steroids and growth factors, in regulating the proliferative activity of OSE (Chapter Three).

(3) To test the hypothesis that pregnancy suppresses EOC by inducing apoptosis within the OSE layer and inclusion cysts, a process mediated through over-expression of *p53* gene, which is under the control of high progesterone and oestrogen level. For the *in situ* tests bovine histological tissues and for *in vitro* analysis cultured OSE cells were used. This is because the EOC is reported in cows but not in sheep (Chapter Four).

(4) To examine a role of pregnancy and of inhibition of pituitary gonadotrophin production by administration of GnRH antagonist, on OSE cells' morphology and activity in a non-human primate, the marmoset monkey. For this, IHC method was selected using BrdU proliferative marker at different stages of the ovulatory cycle and during pregnancy (Chapter Five).

CHAPTER TWO

The Influence of the Reproductive Stage on OSE Proliferation and Follicular Dynamics in Sheep

2.1 Introduction

The ovarian surface epithelium (OSE) forms a lining around the entire ovary and actively participates in the ovulatory cycle. Before ovulation occurs OSE cells close to the rupture site of the follicle undergo apoptotic cell death (Murdoch 1996). It is assumed that the wound caused by ovulation is repaired by proliferating OSE cells (Osterholzer *et al.* 1985). Research in rabbits has shown proteolytic enzymes capable of degrading the basement membrane and apical follicular wall are produced by the OSE (Bjersing & Cajander 1975). The biology of OSE cells is of interest since the majority of human ovarian cancers originate in these cells (follicular granulosa cells, stroma, and germ cells account for the remainder) (Vanderhyden *et al.* 2003).

Epithelial ovarian cancer (EOC) is the fifth most common female cancer world wide and the most lethal gynaecological malignancy (Jackson *et al.* 2009). Cancerous growth in these cells is thought to be associated with high proliferative activity in the OSE cells where multiple ovulations have occurred. Murdoch (2005) hypothesized that repeated cycles of ovulation-induced trauma and repair of the OSE at the site of ovulation contributes to malignancy and may be associated with mitogenic effects of hormones. This hypothesis is supported by *in vitro* experiments where isolated murine OSE cells continuously maintained in proliferative conditions acquired malignant features, including loss of contact inhibition, substrate-independent growth, and the ability to form tumours in nude mice (Roby *et al.* 2000).

Epidemiological data has shown that pregnancy and the use of oral contraceptives can reduce the risk of EOC development (Clow *et al.* 2002). Additionally, it has been reported that EOC is absent in animal species in which seasonal ovulation and multiple pregnancies take place (Fathalla 1971). Although pregnancy is proposed as a protective factor against ovarian cancer, the mechanism by which it works is unknown. During pregnancy, several factors may alter the ovarian physiology and with it the dynamics of follicular growth. Suppression of ovulation and steroid production by the pre-ovulatory follicles may alter the physiological status of the OSE specifically at the area of direct contact with the growing follicles. Therefore, the

influence of pregnancy on OSE may be an indirect effect via changes in growth of ovarian follicles.

Several studies have focused on follicular dynamics during pregnancy (Rizzo *et al.* 2009; De los *et al.* 2006; Ohshima *et al.* 2002; al-Gubory & Martinet 1986), but none of them looked at the effect of pregnancy on the OSE cells. Indeed, as most of the OSE research has been conducted *in vitro,* little data exists regarding the *in vivo* situation.

This study was designed to investigate the relationship between stage of the reproductive cycle, follicle dynamics and OSE activity. The sheep was used as an *in vivo* model since this is a mono-ovular species and a seasonal breeder; therefore it is a suitable model to investigate the proliferative activity of the OSE during a quiescence stage such as anoestrus. Ovulatory cycles in most sheep breeds in the northern hemisphere occur in a regular pattern between autumn and winter with oestrus cycles that range in length from 14 to 18 days (Hafez 1952). Ewes exhibit an anoestrus phase that starts from the end of spring until the start of summer. Ovaries from ewes at different stages of the cycle and during pregnancy can be easily obtained from the local abattoir.

The aims of the work described in this chapter were: 1) to examine the relationship between reproductive stage of the cycle (pregnancy, anoestrus, and oestrus), and the proliferative activity of the OSE and 2) determine whether a relationship exists between OSE activity and its location within the ovary, specifically the influences of growing follicles on OSE cells morphology and activity.

A detailed morphometric analysis to categorise the ovarian follicle population during different stages of the reproductive cycle was carried out. To correlate the pattern of follicle development dynamics with OSE activity, cell proliferation was monitored using immunohistochemistry (IHC) of proliferating cell nuclear antigen (PCNA) and Ki-67 proteins, which are standard methods for detecting proliferating cells in tissue sections (Tomanek & Chronowska 2006; Scholzen & Gerdes 2000).

PCNA is a nuclear protein whose expression is markedly increased during the proliferative phase of the cell cycle (Travali *et al.* 1989). It has a critical role in the process of DNA damage repair, including nucleotide excision repair and mismatch repair (Nichols & Sancar 1992; Umar *et al.* 1996). Another competitive proliferating

marker is Ki-67 whose expression is strictly correlated with the active phase of the cell cycle. The Ki-67 antigen is consistently absent in quiescent cells and is not detectable during the DNA repair processes (McCormick *et al.* 1993).

The hypothesis being tested is that periods of anovulation through either pregnancy or seasonal anoestrus results in down regulation of follicular activity and reduced proliferation of OSE cells.

2.2 Materials and methods

2.2.1 Animals

Adult ewe ovaries from cycling and pregnant animals were collected from a local abattoir. The ovaries were gently handled during collection to avoid cell loss by excessive handling as the OSE is a very fragile layer. Ovaries were then transported in a sterile thermos containing culture media of M199/MCDB 105 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). The ovaries were classified according to the reproductive status of the ewes: 1) ovaries from cycling ewes (n = 6) (visible ovarian activity and corpus luteum (CL) present) in the age range of 14–18 months were collected during February (breeding season), 2) ovaries from anoestrus ewes (n = 5) (absence of the follicular activity and no CL) were collected during June (non-breeding season), and 3) ovaries from ewes with an identifiable pregnancy (presence of the foetus in the uterine horn). This last group was further sub-divided into two groups based on the stage of pregnancy using foetal crown-rump length to determine the stage of pregnancy (Sivachelvan *et al.* 1996). The two groups were as follows: ovaries in early-mid gestation (30–75 days) (n = 8) and ovaries in mid-late gestation (80–135 days) (n = 9), full term pregnancies range from 144–155 days. Material from pregnant animals was collected alongside material from cycling animals, and only ovaries from singleton pregnancies were used for this study.

2.2.2 Histological methods

Ovaries were processed for histological analysis by fixing them in Bouin's solution. They were then transferred through 70 to 100 percent graded alcohols, cleared in cedar wood oil, and embedded in paraffin (McCormack Scientific, St Louis, Mo). Wax mounted ovaries were serially sectioned at 6 µm using a microtome (Leica, model Jung RM2035, Nussloch, Germany). The sections were mounted on SuperFrost plus microscope slides (VWR International Ltd., Leicestershire, UK) and left to dry overnight at 37 ºC.

For the morphology and morphometric study, analysis was done on every tenth section with sections in between left aside for IHC. Sections were dewaxed and

rehydrated (100 to 70%) using descending concentrations of ethanol. The sections were stained with eosin (Sigma Chemicals, Poole, Dorset, UK) and haematoxylin (BDH Laboratory Supplies, Poole, UK), dehydrated through ascending series of ethanol. After placing the sections in xylene, DPX (BDH Laboratory Supplies, Poole, UK) was applied as a mounting medium. Sections were examined microscopically using a light microscope (ML2300/T/S Meiji) fitted with an ocular micrometer and digital video camera (JVC TK-1381 CCD) for image capture.

2.2.3 Follicle classification

Ovarian follicular distribution during the different reproductive stages was studied by classifying and counting each stage of follicle development in each group. Morphological observations were carried out on every tenth section. Follicles were classified according to criteria described by McNatty *et al.* (1999) as primordial, transitory, primary, preantral, and antral (Table 2.1). In the classification system for ovine follicles used in this study, the follicles are classified as a primordial follicle when the oocyte is surrounded by one layer of flattened granulosa cells (GCs); a transitory follicle when the oocyte is surrounded by one layer of flattened and cuboidal GCs; a primary follicle when the oocyte is surrounded by one or two layers of cuboidal GCs; a preantral follicle when the oocyte is surrounded by four to six layers of GCs thereby making the follicle variable in size; and an antral follicle when the oocyte is surrounded by more than six layers of GCs and has a visible antral cavity (Figure 2.1).

To avoid counting the same follicle more than once through the sections, only follicles with a clearly visible oocyte nucleolus were counted. An ocular micrometer was used to measure the diameters of antral follicles. Two measurements for each antral follicle (theca layers were excluded) were averaged and expressed as a follicle diameter.

Table 2.1

Classification and characterisation of ovine follicles

Follicle type	Layers of GCs cells	Presence of theca (%)
Primordial	1 layer of flattened cells	0
Transitory	1 layer of flattened and cuboidal cells	0
Primary	1–2 layers of cuboidal cells	35
Preantral	4-6 layers	100
Antral	6+ layers with the appearance of antrum	100

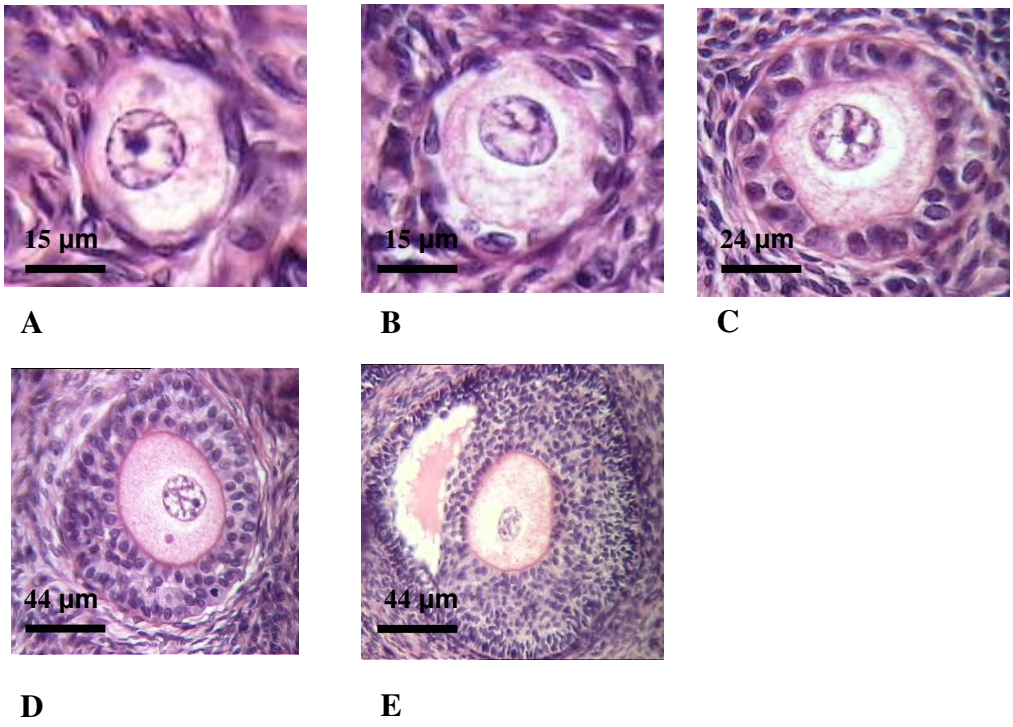


Figure 2.1 Histological representation of ovine follicles at different stages of development: (A) primordial, (B) transitory, (C) primary, (D) preantral and (E) antral.

2.2.4 *Quantification of ovarian follicles*

Due to the large size of the ewe ovary (~1.5 x 1 x 0.8 cm) follicle counts were carried out using a sampling method. To determine whether sampling from one region of the ovary was representative of others, the whole ovary was divided into three parts, and each part was analyzed individually (Figure 2.2). The exact number of follicles cannot be assessed reliably using this sampling technique, but will enable a sufficiently accurate representation of the relative proportions of different sized follicles to be estimated.

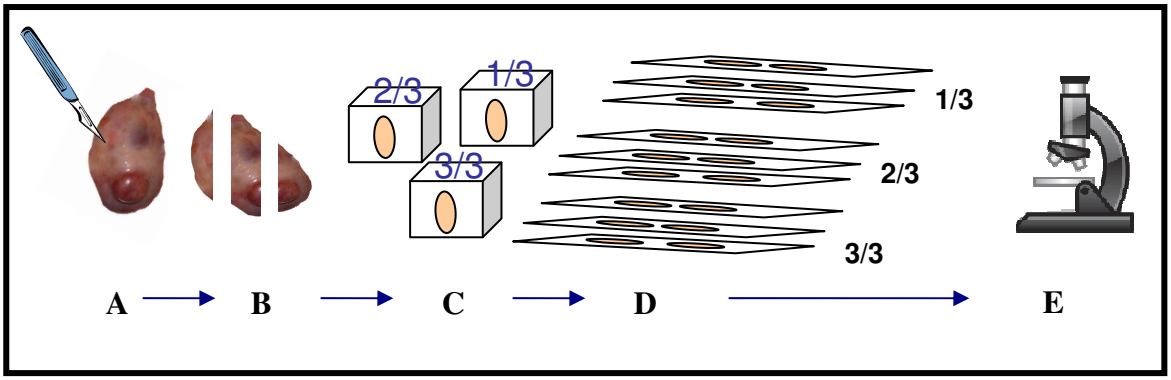


Figure 2.2 Sampling method to estimate ovarian follicle distribution per ovary. A sampling technique was performed to determine whether a part (one-third) of the ovary is representative of the distribution of ovarian follicles. One whole ovary from each group of the study (cycling, anoestrus, and pregnant) was studied. (A) One ovary was chosen randomly from each group, (B) selected ovaries were divided longitudinally into three equal parts, (C and D) each part was processed for histology (sectioned at 6µm) and (E) microscopic analysis.

The result from Kruskal-Wallis test ($P< 0.05$) revealed that the distribution of follicular stages was representative throughout the three parts of the ovary tested; therefore, only one part of the ovary was selected for subsequent analysis of the remaining ovaries. Counting was carried out for every tenth section for each of the three parts using a light microscope (Table 2.2, 2.3, and 2.4).

Table 2.2

Ovarian follicle distribution from cycling ewes

Follicle stage	1/3		2/3		3/3	
	Total number of follicles	**%**	**Total number of follicles**	**%**	**Total number of follicles**	**%**
Primordial	1152	39.0	655	25.0	238	35.0
Transitory	1617	55.0	1758	67.0	419	61.0
Primary	125	5.0	182	7.0	19	3.0
Preantral	23	0.8	14	0.5	7	0.9
Antral	13	0.4	11	0.4	6	0.1

Table 2.3

Ovarian follicle distribution from anoestrus ewes

Follicle stage	1/3		2/3		3/3	
	Total number of follicles	**%**	**Total number of follicles**	**%**	**Total number of follicles**	**%**
Primordial	2301	57.9	2061	58.0	2019	56.0
Transitory	1562	39.4	1393	39.0	1478	41.0
Primary	85	2.1	81	2.2	84	2.3
Preantral	7	0.2	9	0.3	11	0.2
Antral	15	0.4	18	0.5	17	0.5

Table 2.4

Ovarian follicle distribution from pregnant (mid-late gestation; 80–135 days) ewes

Follicle stage	1/3		2/3		3/3	
	Total number of follicles	**%**	**Total number of follicles**	**%**	**Total number of follicles**	**%**
Primordial	2464	27.0	1504	33.0	2633	36.0
Transitory	6253	69.0	2816	62.0	4558	61.0
Primary	289	3.0	158	4.0	191	3.0
Preantral	36	0.4	21	0.5	32	0.4
Antral	24	0.2	11	0.2	19	0.2

2.2.5 PCNA and Ki-67 immunohistochemistry

The effect of the reproductive stage (oestrus, anoestrus or pregnancy) on proliferative activity of the OSE and GCs was investigated using IHC to PCNA and Ki-67 in order to quantify proliferating cells. Sections were de-waxed in xylene, and hydrated in descending grades of alcohol to distilled water, and followed by double washing with phosphate buffer saline (PBS). A heat-induced epitope retrieval method was performed (antigen retrieval) by microwaving (high power at 800W) the sections in a 0.01M citrate buffer (pH 6.0) for 10 min, and letting them stand in hot buffer for a further 20 min. Endogenous peroxidase activity was inactivated through incubation with 3 percent hydrogen peroxide in methanol. An Invitrogen PCNA staining kit was used for PCNA detection (93-1143, Invitrogen Corporation, Camarillo). All the reagents included in the kit (blocking solution, biotinylated mouse anti- rat PCNA monoclonal antibody, streptavidin-peroxide, and 3,3′-Diaminobenzidine tetrahydrochloride (DAB) chromogen were ready prepared. Sections were incubated with blocking solution for 10 min followed by overnight incubation with the primary antibody (biotinylated mouse anti-PCNA clone PC10) at 4° C. After rinsing with PBS, sections were incubated for 10 min with streptavidin-peroxide complex at room temperature. Following the PBS wash, DAB

solution was added to the sections for between 5 and 10 min. After being washed with tap water, sections were counterstained with hematoxylin for 25 sec, dehydrated in graded alcohol, cleared, and then mounted with DPX.

Ki-67 lyophilized rabbit anti-human polyclonal antibody (NCL-Ki 67p, Novocastra Laboratories Ltd., UK) was used for Ki-67 detection. Antigen retrieval and endogenous peroxidase activity blocking were performed as described previously (PCNA staining). The NovoLink polymer detection system (RE7 150-K, Novocastra Laboratories Ltd., UK) was used for visualization. Sections were incubated with protein block for 5 min, followed by an overnight incubation with the primary antibody (1:2000 at 4° C). After being washed with PBS, a post-primary block was performed for 30 min. Sections were incubated with the NovoLink polymer solution for 30 min, and then washed with PBS. Peroxidase activity was developed using a DAB solution for 5 min. Sections were counterstained using hematoxylin stain. Further optimization of Ki-67 antibody was necessary to enhance the permeability to the tissue. Antibody incubation time and temperature were adjusted to overnight at 4° C instead of 60 min at 37° C. For PCNA, we increased the washing step to three times in order to reduce background.

As a negative control, the blocking solution was left on the sections instead of the primary antibody to insure the purity of the staining procedure (Figure 2.3). Sheep tonsil was used as a positive control. At each run of IHC, a set of slides including sections from all groups of study plus negative and positive control were run together under the same experimental condition. Three sections from each ovary were selected randomly for the analysis (for each antibody). Immunostaining was examined using a light microscope, and images were captured with a digital video camera.

2.2.6 Quantification of PCNA and Ki-67 immunostaining

PCNA and Ki-67 staining was quantified by counting OSE and GCs (positive or negative). All of the OSE cells overlying the ovary in the circumference of the section were counted at 400X magnification. The number of positive nuclei of all counted cells was used to calculate the percentage of proliferation. The proliferation index for large antral follicles (larger than 750 μm) was assessed by counting at least 400 GCs/area

from four different areas around the follicle using an eyepiece graticule (cross lines) at 200X magnification.

2.2.7 Statistical analysis

Immuohistochemistry results for Ki-67 and PCNA labelling in OSE and GCs were expressed as a mean percentage of labelled cells. The total number of cells (labelled and unlabelled) was counted for each ovary. Data for proliferative cells distribution at different regions of the OSE was expressed as a total percentage of labelled cells at each region. Results of follicle distribution at different stages of development (primordial, transitory, primary, preantral and antral) were expressed as a mean percentage of each type per ovary. Data for antral follicle sizes were demonstrated as total percentages of antral follicle size per group. Data was not normally distributed in accordance with the Kolmogorov-Smirnov test and box plot graphs, therefore, Kruskal-Wallis test was selected as a useful test for comparing more than two groups. The Mann-Whitney test was applied in order to compare two groups (Ki-67 and PCNA labelling in OSE cells). Chi-square test was used to compare the total percentages between groups (distribution of OSE proliferative cells and antral follicles size variation). Differences were considered to be significant at $P \leq 0.05$. Analysis was done using Minitab version 15.

2.3 Results

2.3.1 PCNA and Ki-67 immunostaining in OSE cells

PCNA and Ki-67 IHC was performed on serial sections of ewe ovaries. In these sections, PCNA and Ki-67 positive cells showed brown nuclear staining. The photomicrographs in Figure 2.3 show the positive and negative immunostaining for Ki-67 and PCNA in antral and preantral follicles. Ki-67 and PCNA both show a high level of expression when compared to the OSE cells.

There was no evidence of labelling for either marker PCNA or Ki-67 in the OSE from pregnant and anoestrus groups since no stained nuclei within the OSE cells could be detected (Figure 2.4). Positive immunostaining was identified only in the OSE layer of cycling ewes, 0.53 ± 0.05% for PCNA and 0.30 ± 0.07% for Ki-67 (Figure 2.5). High staining for PCNA was observed in OSE invagination, these cells were cuboidal in shape. Although the percentage of both markers was very low, the PCNA expression was significantly higher than the Ki-67 (P= 0.02).

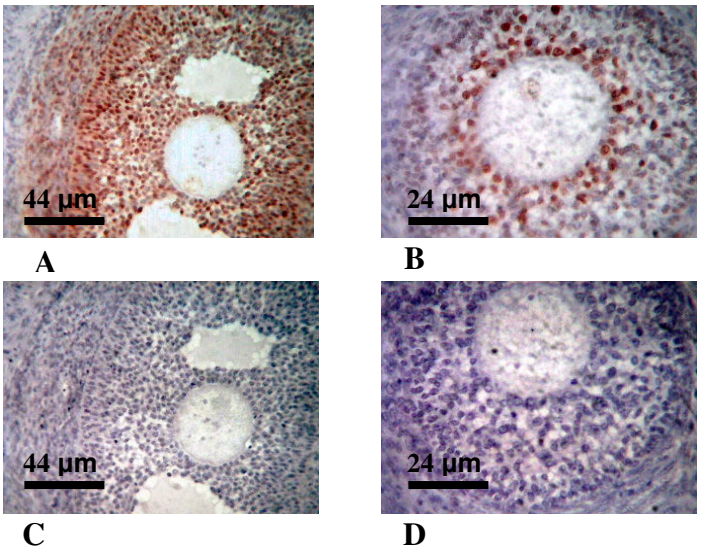


Figure 2.3 Photomicrographs showing the immunohistochemical staining for (A) PCNA and (B) Ki-67 in GCs of ovine growing follicles. Panels (C) and (D) demonstrate the negative controls of the same follicles.

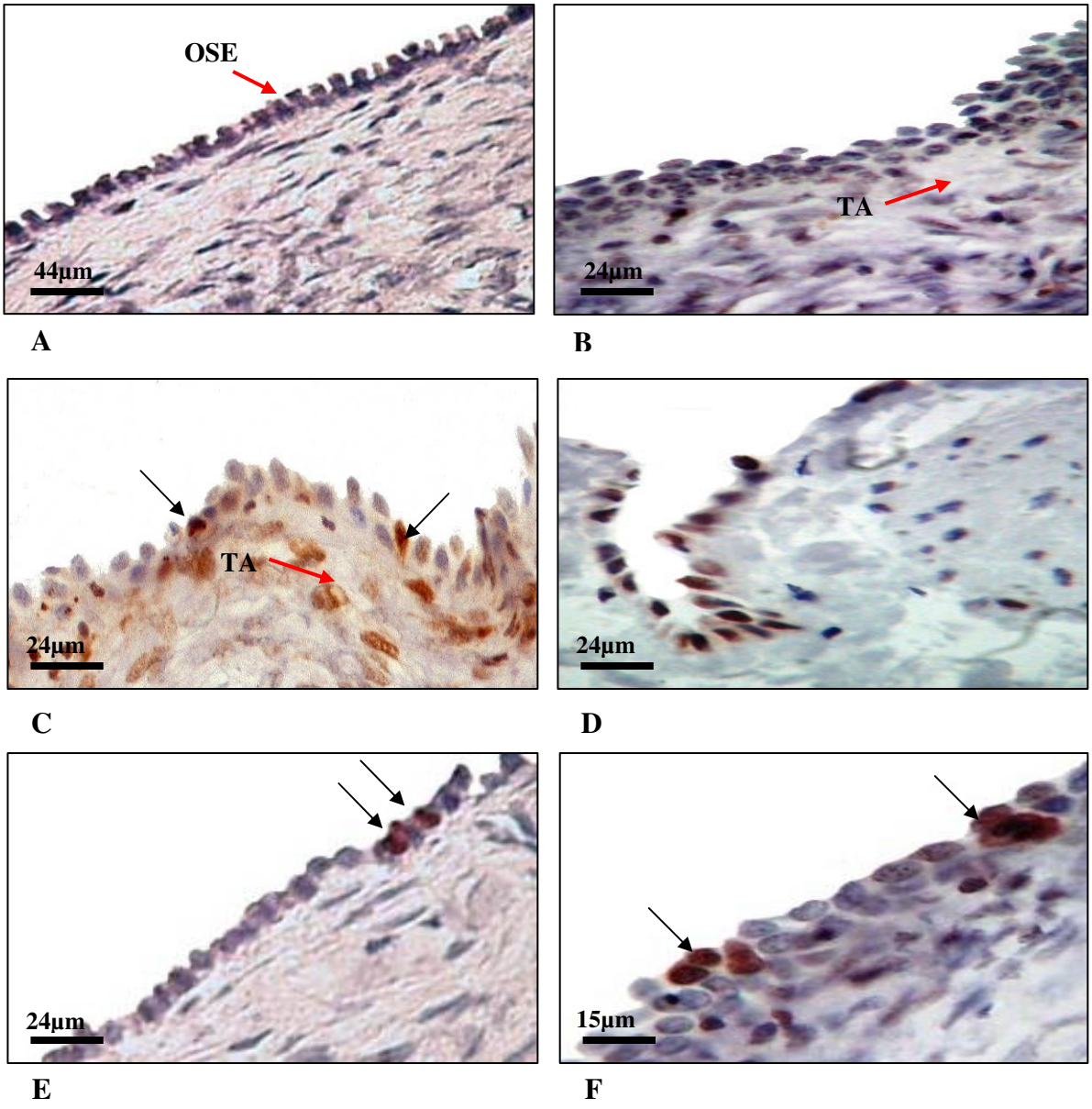


Figure 2.4 Photomicrographs showing PCNA and Ki-67 immunostaining in the OSE layer. No detectable staining for either marker was observed in OSE cells in (A) anoestrus or (B) pregnant groups (TA: tunica albuginea). Panels C through E demonstrate PCNA localization in OSE cells in the (C) cycling group, at (D) OSE invagination, and (E) over the ovarian stroma in close proximity to large antral follicles (arrows). Panel F shows Ki-67 staining in OSE cells over a large antral follicle in the cycling group. Black arrows indicate the proliferating cells (brown stain).

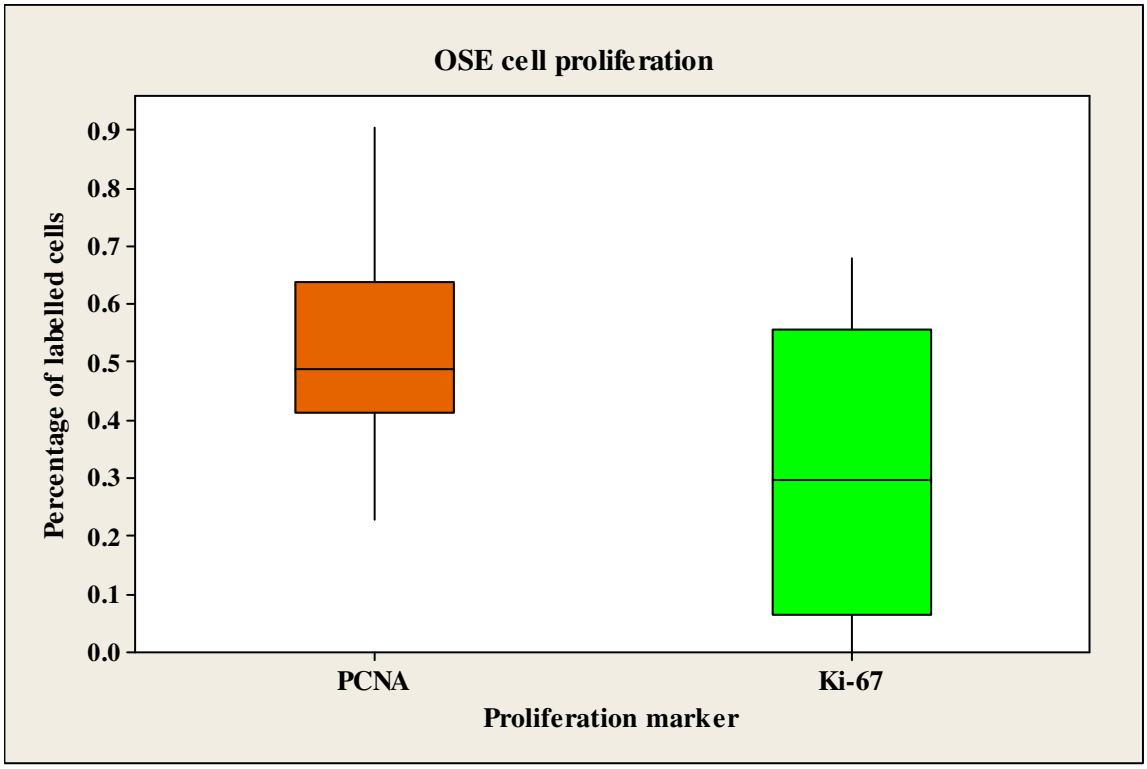


Figure 2.5 Box plots representing OSE cell proliferation as indicated by PCNA and Ki-67 immunohistochemistry of cycling ewes (n = 6). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles. Data are the percentage of labelled cells for each section (18 sections/ marker). Total number of counted OSE cells over the total area of the surface is presented in Table 2.5.

Table 2.5

PCNA and Ki67 labelling in OSE cells of cycling ewes

Animal	PCNA labelling			Ki-67 labelling		
	No. labelled cells	No. unlabelled cells	% of labelling	No. labelled cells	No. unlabelled cells	% of labelling
1	15	1891	0.79	2	1214	0.25
1	9	1771	0.60	1	1009	0.10
1	10	1710	0.58	5	911	0.55
2	5	1327	0.38	0	1152	0.26
2	9	1219	0.73	0	979	0.20
2	5	1109	0.45	4	1201	0.33
3	13	1659	0.42	0	992	0.00
3	8	1210	0.33	7	1216	0.57
3	7	1398	0.50	0	1165	0.00
4	9	1425	0.63	4	1076	0.37
4	7	1502	0.46	4	995	0.40
4	6	1279	0.47	6	1108	0.63
5	11	1677	0.90	0	1092	0.00
5	5	1814	0.38	0	1184	0.00
5	5	1312	0.23	5	1119	0.44
6	8	1430	0.56	1	1175	0.09
6	11	1643	0.67	7	1002	0.68
6	9	1447	0.48	2	1023	0.59

2.3.2 Distribution of OSE proliferating cells in cycling group

Analysis of PCNA staining over different regions of the OSE revealed that the majority of PCNA and Ki-67-stained OSE cells were localised over the corpora lutea (44 and 52%) of the total labelled OSE cells, and over large antral follicles (2.5–3.5 mm in diameter) (39 and 40%). A much lower fraction (17 and 8%; P≤ 0.05) was found over the stroma (Figure 2.6).

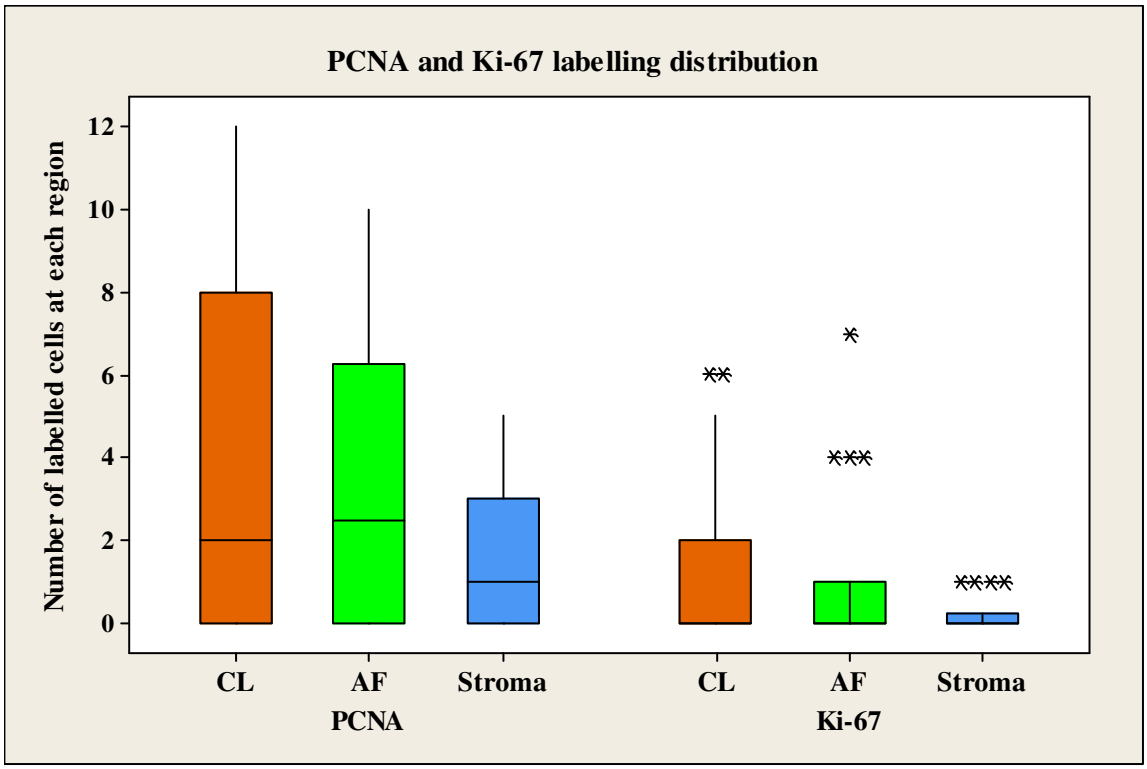


Figure 2.6 Box plots representing the distribution of proliferating cells in OSE of cycling ewes (n = 6) at different regions around the ovary. PCNA and Ki-67 labelling in the OSE layer was examined overlying the corpora lutea (CL), antral follicles (AF) and stroma (devoid of follicles or corpora lutea). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Data are the number of OSE labelled cells at each region (3 sections/ animal). The total number of counted OSE cells over the three different regions of the ovary is presented in Table 2.6.

Table 2.6

Distribution of PCNA and Ki-67 labelling within the OSE cells at different regions around the ovary

Animal	PCNA labelling				Ki-67 labelling			
	Total labelled cells	**CL**	**AF**	**Stroma**	**Total labelled cells**	**CL**	**AF**	**Stroma**
1	15	12	0	3	2	1	0	1
1	9	6	0	3	1	0	0	1
1	10	10	0	0	5	5	0	0
2	5	0	3	2	0	0	0	0
2	9	0	9	0	0	0	0	0
2	4	0	2	2	4	0	4	0
3	13	0	8	5	0	0	0	0
3	8	0	7	1	7	0	7	0
3	7	5	0	2	0	0	0	0
4	9	9	0	0	4	0	4	0
4	7	4	0	3	4	0	4	0
4	6	0	6	0	6	6	0	0
5	11	8	3	0	0	0	0	0
5	5	5	0	0	0	0	0	0
5	5	0	5	0	5	5	0	0
6	8	8	0	0	1	1	0	0
6	11	0	10	1	7	6	0	1
6	9	0	5	4	2	1	0	1

2.3.3 Morphology of the OSE cells at different regions around the ovary

There was variation in the cellular morphology of the OSE cells that correlated to location. The OSE layer over large antral follicles was composed of flattened squamous cells with a loose attachment to the fragmentary basement membrane. As is typically found, the OSE over corpora lutea was a monolayer of simple cuboidal epithelial cells with dilated intracellular spaces in between an undetectable basement membrane. Away from the active site of ovulation, over the stroma, the OSE exhibited an organized order of compact stratified cuboidal cells with a distinguishable basement membrane (Figure 2.7). The OSE layer in the pregnant group showed a stratification structure of 2-3 layers of cells, none of which were positively stained with PCNA or Ki-67.

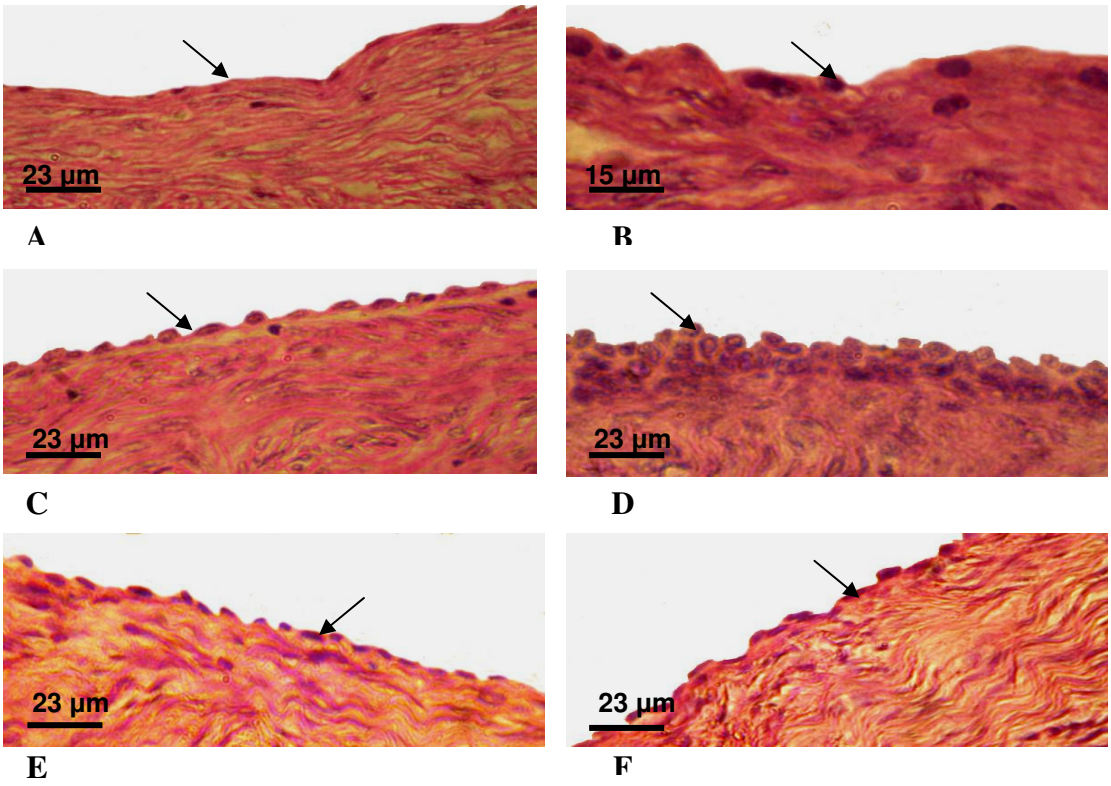


Figure 2.7 Photomicrographs showing the morphology of the OSE layer (stained with hematoxylin and eosin). Panels A through C demonstrate the morphology of the OSE layer of cycling ewes: (A) shows a thin incomplete monolayer of flat cells overlying a large antral follicle, (B) an incomplete monolayer of cuboidal cells covering the CL and (C) a monolayer of organized flat to cuboidal cells over the stroma. Panel D shows complete and well-organized cells in the pregnant group. Panels E and F demonstrate the morphology of the OSE layer of flat to cuboidal cells in the anoestrus group.

2.3.4 PCNA and Ki-67 immunostaining in the GCs of growing follicles

Nuclear staining for PCNA in GCs of all types of follicles was higher than Ki-67 labelling, which was negligible in some of the follicular GCs. Surprisingly, PCNA labelling was detected in oocytes of the early stages of follicle development (primordial and transitory follicles) (Figure 2.8). No detectable Ki-67 staining was observed in primordial or transitory follicles in any of the groups. Neither stained oocytes nor GCs within these follicles were observed. However, there was variation between groups, with the highest rate of labelling observed in the follicular GCs of cycling ewes (Table 2.7, page 46). Ki-67 was observed in GCs of follicles from the primary stage and beyond with variation between groups (Figure 2.9 and 2.10) (Table 2.8, page 47). The expression of Ki-67 was statistically lower than PCNA in GCs of all types of follicles (Figure 2.11).

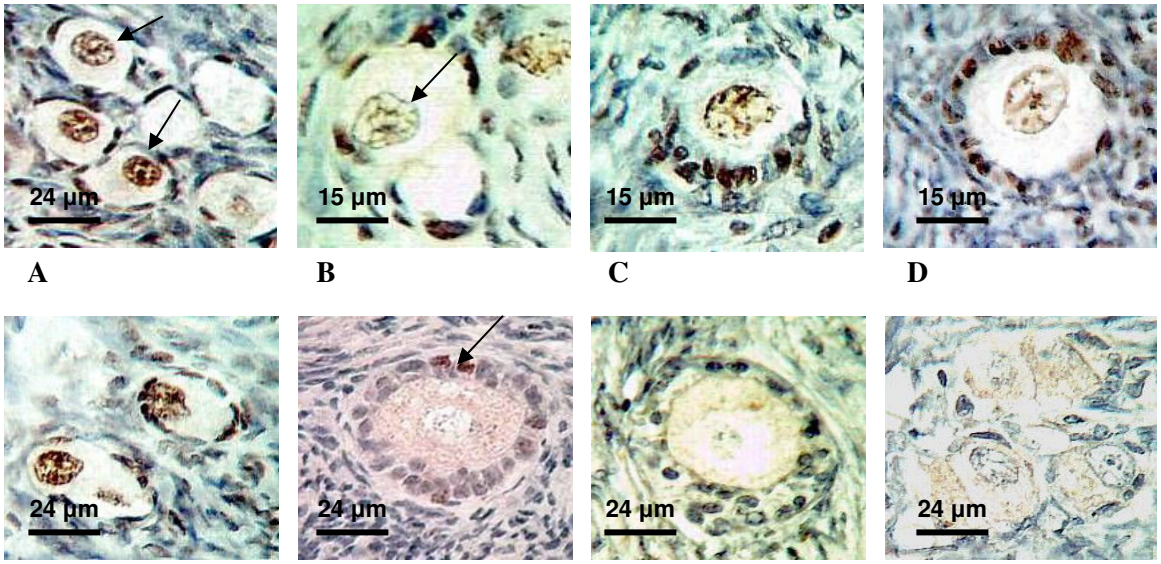


Figure 2.8 Photomicrographs showing immunohistochemical staining of PCNA and Ki-67 in GCs of ovarian follicles. Panels A through E demonstrate PCNA labelling in GCs and oocytes of primordial follicles in (A) cycling (arrows show oocyte labelling) and (B) pregnant groups (arrows show unlabelled oocyte and labelled GCs); (C) transitory; (D) primary follicles in cycling group; and (E) primordial follicles in anoestrus group. Panels F and G demonstrate Ki-67 labelling in cells of primary follicle (F) in cycling and (G) anoestrus groups. (H) Transitory follicles in the pregnant group are also shown (no detectable staining).

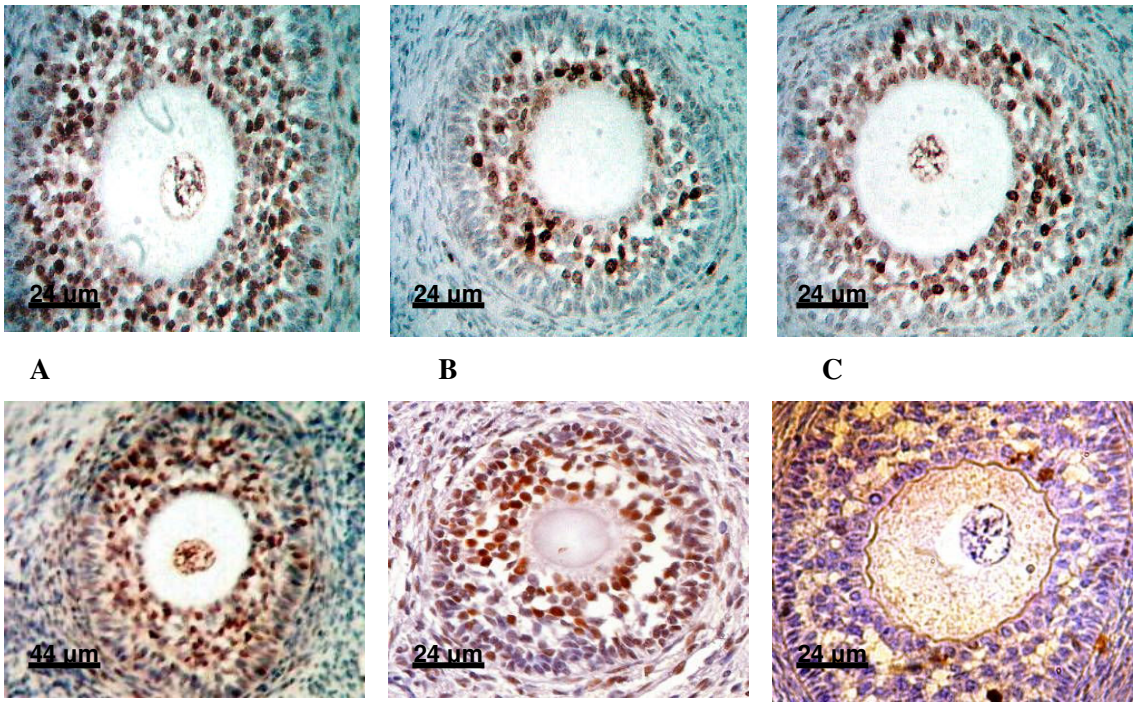
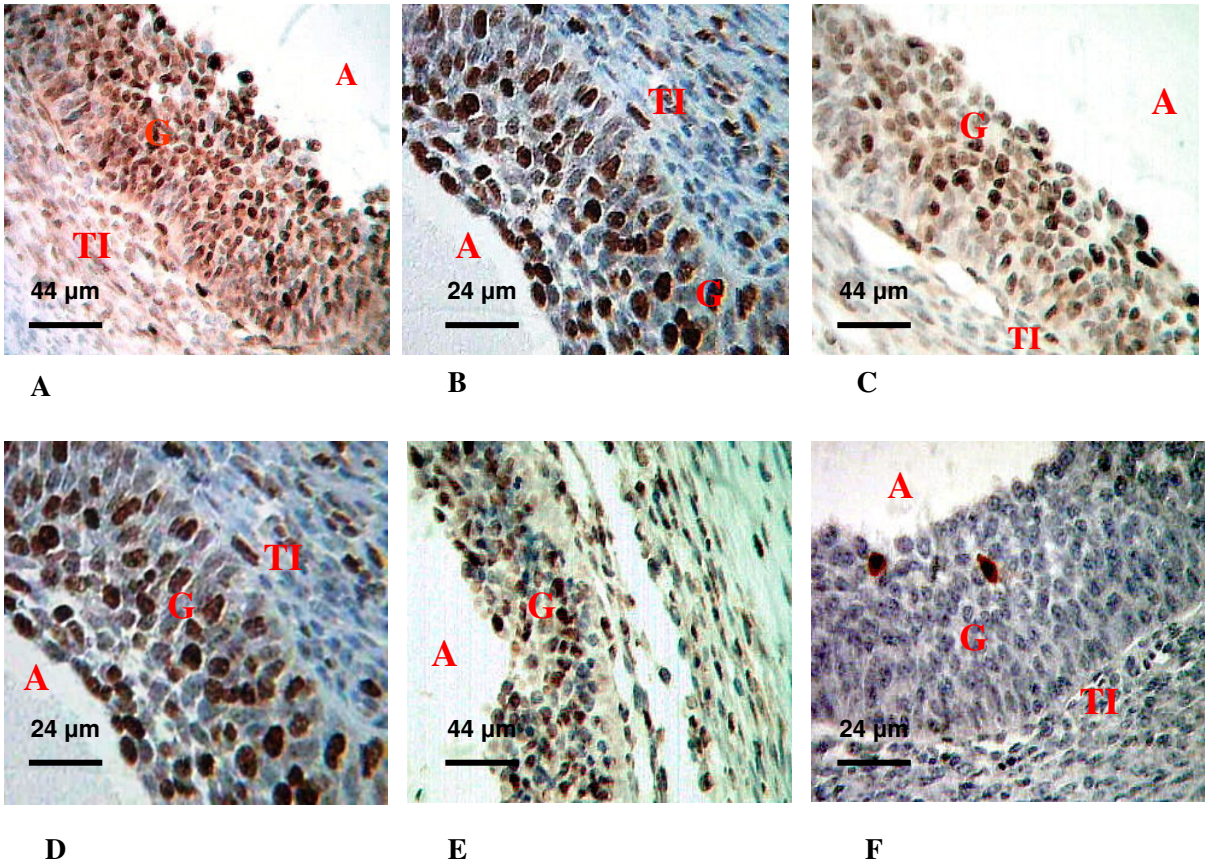


Figure 2.9 Photomicrographs showing immunohistochemical staining of PCNA and Ki-67 in GCs of preantral follicles. Panels A through C demonstrate PCNA labelling of preantral follicles in (A) cycling, (B) anoestrus, and (C) pregnant groups. Panels D through F demonstrate Ki-67 labelling in the GCs of preantral follicles in (D) cycling, (E) anoestrus and (F) pregnant groups.



A **B** **C**

D **E** **F**

Figure 2.10 Photomicrographs showing immunohistochemical staining of PCNA and Ki-67 in GCs of antral follicles. Panels A through C demonstrate PCNA labelling in GCs of antral follicles in (A) cycling, (B) anoestrus and (C) pregnant groups. Panels D through F demonstrate Ki-67 labelling in the GCs of (D) cycling, (E) anoestrus and (F) pregnant groups. G, granulosa layer; A, antrum; TI, theca interna.

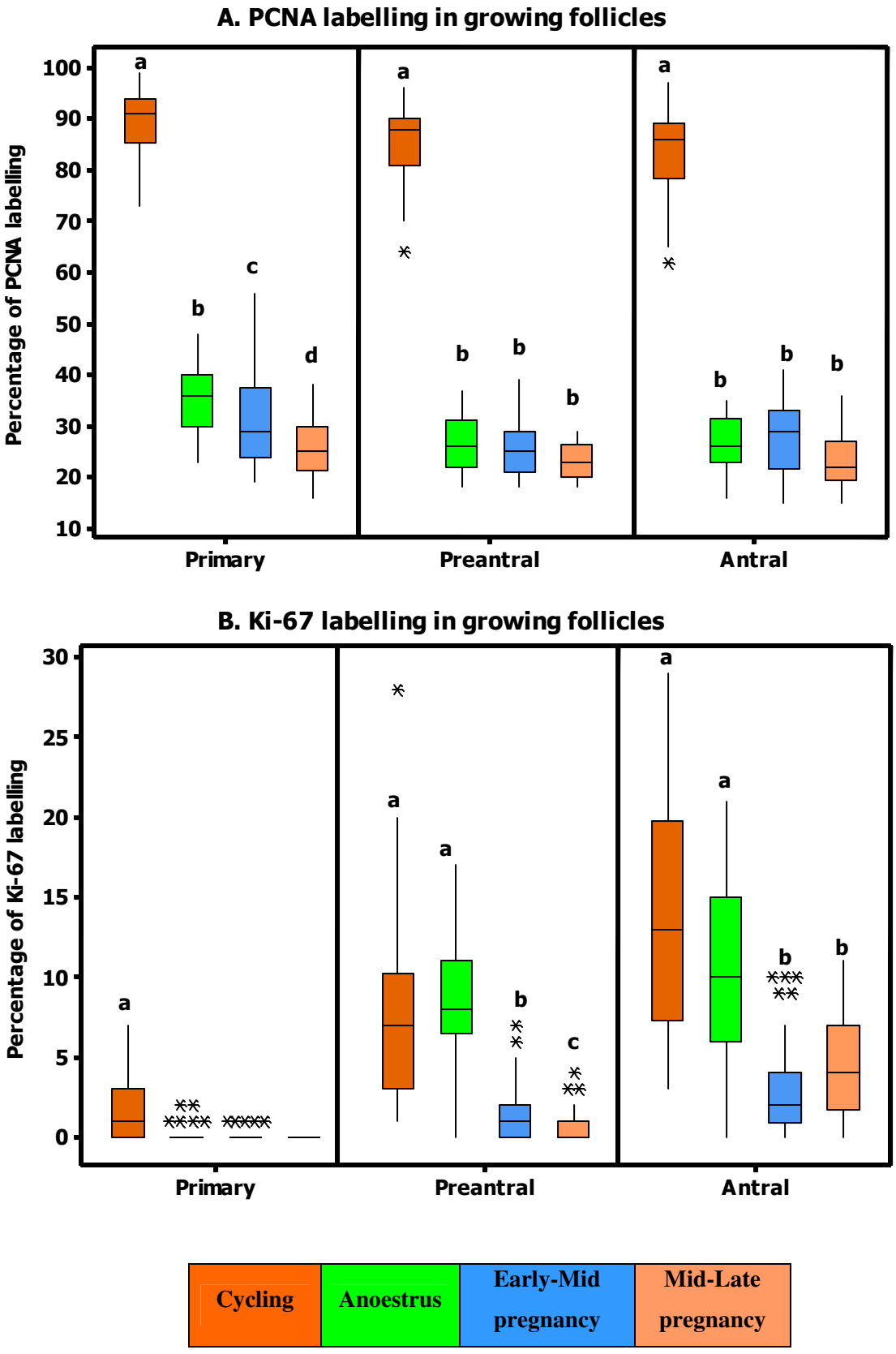

A. PCNA labelling in growing follicles
Percentage of PCNA labelling
100
90
80
70
60
50
40
30
20
10
a
b
c
d
a
b
b
b
a
b
b
b
Primary
Preantral
Antral
B. Ki-67 labelling in growing follicles
Percentage of Ki-67 labelling
30
25
20
15
10
5
0
a
a
a
b
c
a
a
b
b
Primary
Preantral
Antral
Cycling
Anoestrus
Early-Mid pregnancy
Mid-Late pregnancy

Table 2.7

PCNA immunostaining in the GCs of growing follicles

Group	Primary		Preantral		Antral	
	Number of follicles	**Mean percentages of labelling/ follicle ±SEM**	**Number of follicles**	**Mean percentages of labelling/ follicle ±SEM**	**Number of follicles**	**Mean percentages of labelling/ follicle ±SEM**
Cycling (*n* = 6)	69	87.17±0.76	25	85.65±1.61	25	83.41±1.78
Anoestrus (*n* = 5)	29	31.21±0.84	26	26.70±1.16	21	26.94±1.32
Early-mid pregnancy (*n* = 8)	41	28.58±0.80	33	24.97±0.89	29	28.19±1.39
Mid-late pregnancy (*n* = 9)	39	24.03±0.83	15	23.29±0.93	16	23.54±1.67

Table 2.8

Ki-67 immunostaining in the GCs of growing follicles

Group	Primary		Preantral		Antral	
	Number of follicles	**Mean percentages of labelling/ follicle ±SEM**	**Number of follicles**	**Mean percentages of labelling/ follicle ±SEM**	**Number of follicles**	**Mean percentages of labelling/ follicle ±SEM**
Cycling (*n* = 6)	26	1.62±0.32	24	8.05±1.41	22	14.15±1.83
Anoestrus (*n* = 5)	37	0.23±0.10	35	8.58±0.79	35	10.18±0.89
Early-mid pregnancy (*n* = 8)	31	0.18±0.10	45	1.48±0.26	46	2.91±0.94
Mid-late pregnancy (*n* = 9)	49	0	35	0.77±0.18	30	4.41±0.64

2.3.5 Pattern of follicle development at different reproductive status

In order to investigate the pattern of follicle distribution at different reproductive stages, follicles at different developmental stages (primordial, transitory, primary, preantral, and antral) in cycling, anoestrus, and pregnant ewes were counted. The results are outlined in Table 2.9 and 2.10 (Page 50). The Kruskal-Wallis test revealed significant differences between the groups (Figure 2.12), as detailed below:

Primordial and transitory follicles. There was a significant increase in the mean percentages of primordial follicles between anoestrus and all other studied groups and a consequent decrease in transitory follicles. No difference was determined between cycling and early-mid pregnancy groups. In the mid-late pregnancy group, the percentage of primordial follicles decreased and transitory follicles increased significantly.

Primary and preantral follicles. The mean percentages of primary and preantral follicles in the cycling group were significantly higher than in the other groups. In the mid-late pregnancy group, a significant increase was observed when compared to the anoestrus and early-mid pregnancy groups.

Antral follicles. No difference was detected between the cycling and the anoestrus groups in the mean percentages of antral follicles. The number of antral follicles decreased significantly in the early and late stages of pregnancy.

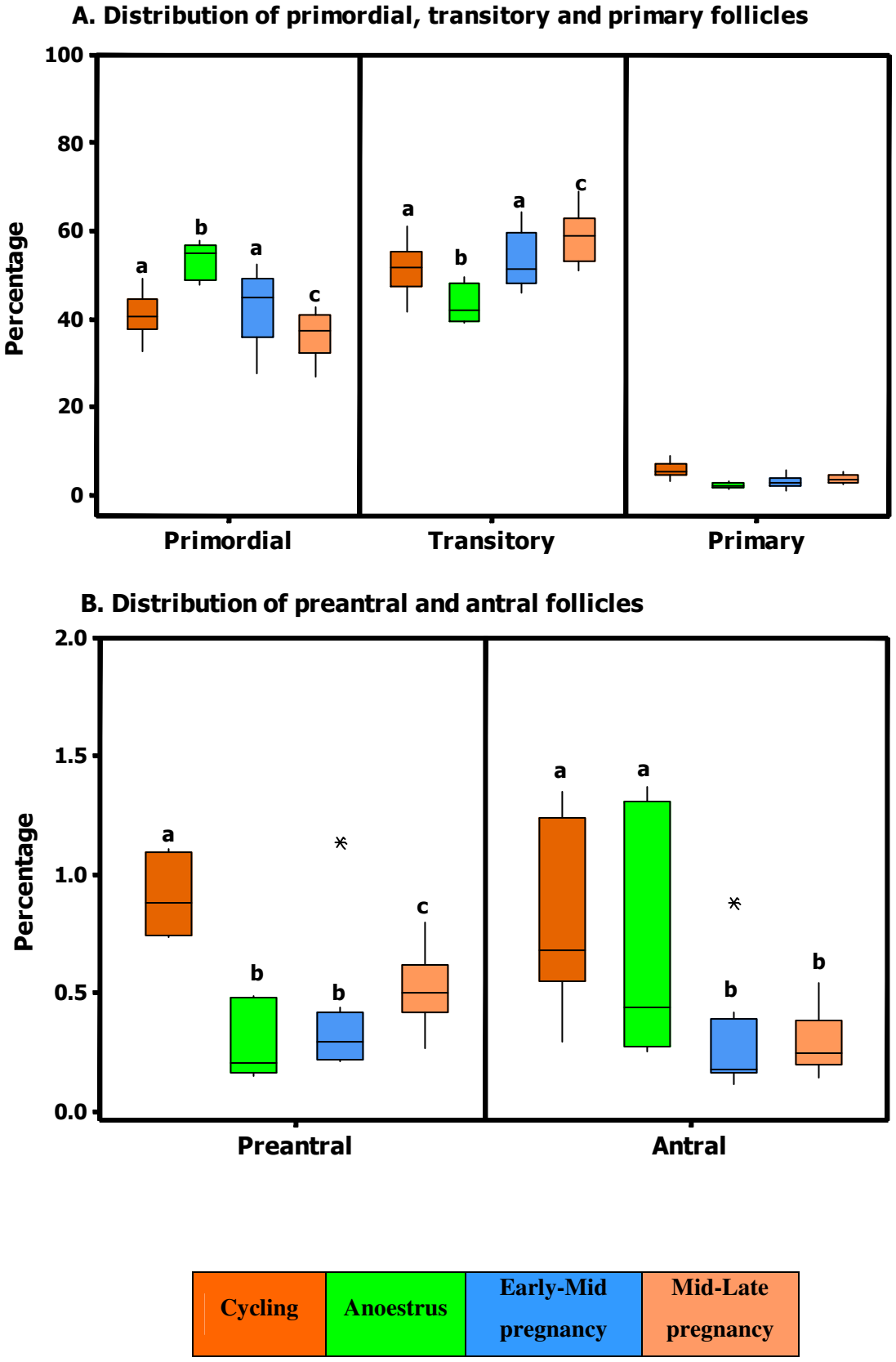

A. Distribution of primordial, transitory and primary follicles
Percentage
100
80
60
40
20
0
a
b
a
c
a
b
a
c
Primordial
Transitory
Primary
B. Distribution of preantral and antral follicles
Percentage
2.0
1.5
1.0
0.5
0.0
a
b
b
c
a
a
b
b
Preantral
Antral
Cycling
Anoestrus
Early-Mid pregnancy
Mid-Late pregnancy

Table 2.9

Distribution of ovarian follicles in cycling and anoestrus groups

Follicle stage	Cycling (*n* = 6; 366 sections)		Anoestrus (*n* = 5; 234 sections)	
	Total number of follicles	Mean percentages ±SEM	Total number of follicles	Mean percentages ±SEM
Primordial	12321	41.30 ± 2.20	13514	53.90 ± 1.59
Transitory	15858	51.61 ± 2.42	10797	43.10 ± 1.75
Primary	1565	5.60 ± 0.68	527	2.00 ± 0.25
Preantral	245	0.85 ± 0.07	61	0.30 ± 0.06
Antral	216	0.80 ± 0.13	152	0.70 ± 0.22

Table 2.10

Distribution of ovarian follicles in pregnant groups

Follicle stage	Early-mid pregnancy (*n* = 8; 391 sections)		Mid-late pregnancy (*n* = 9; 457 sections)	
	Total number of follicles	Mean percentages ±SEM	Total number of follicles	Mean percentages ±SEM
Primordial	23783	42.00 ± 3.03	17791	36.39 ± 1.72
Transitory	27733	54.00 ± 2.44	30671	58.97± 1.93
Primary	1454	3.00 ± 0.52	1770	3.84 ± 0.34
Preantral	177	0.40 ± 0.11	250	0.52 ± 0.05
Antral	133	0.30 ± 0.10	136	0.29 ± 0.04

2.3.6 Classification of antral follicle sizes

The total number and percentage of antral follicles at different sizes are described in Tables 2.11 and 2.12. No significant difference was detected in the percentage of antral follicles ≥ 1 mm and < 3 mm between groups. The number of antral follicles ≥ 3 mm and < 4 mm decreased significantly in early-mid pregnancy and were completely absent in mid-late pregnancy. Antral follicles larger than 4 mm in diameter were not observed at all stages of pregnancy. In contrast, a significant increase was detected in the percentage of 4–5 mm antral follicles in the anoestrus group when compared to the cycling group (Figure 2.13).

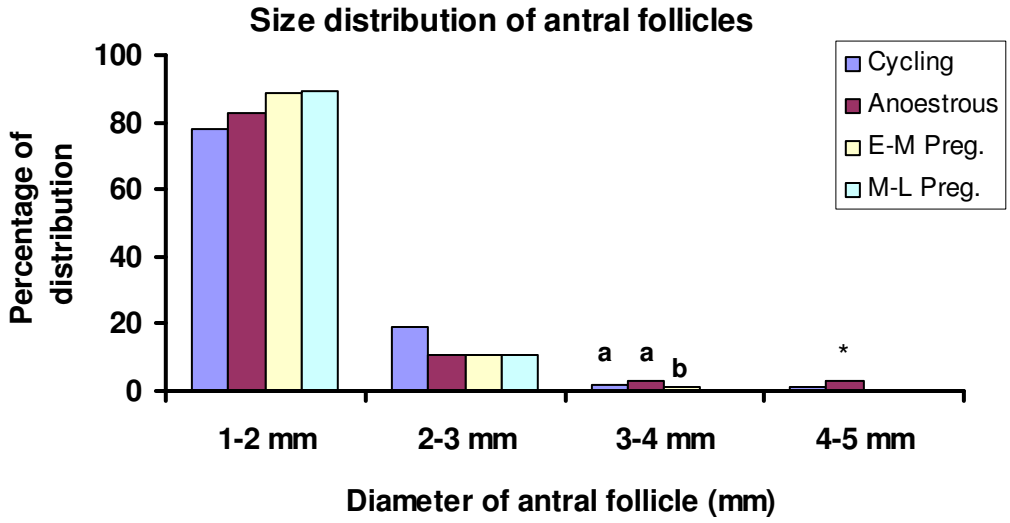


Figure 2.13 Antral follicles size distribution at different reproductive status. Distribution of antral follicles with different diameters ≥ 1 mm, 2-3mm, 3-4mm and 4-5mm at cycling (n = 6), anoestrus (n = 5), early-mid pregnancy (E-M Preg.) (30-75 days) (n = 8), and mid-late pregnancy (M-L Preg.) (80–135 days) (n = 9). Values are total percentages of each size of antral follicle per group. Different letters indicate statistical differences between groups among antral follicles (size 3-4mm) and an asterisk over the bar indicates statistical differences between groups among antral follicles (4-5mm) ($P \leq 0.05$). The total number of analyzed follicles is outlined in Table 2.11 and 2.12.

Table 2.11

Total number and percentages of antral follicles of different sizes in cycling and anoestrus groups

Follicle diameter	Cycling (*n* = 6; 366 sections)		Anoestrus (*n* = 5; 234 sections)	
	Total number of follicles = 216	%	Total number of follicles = 152	%
< 2 mm	167	77.31	82.89	82.89
2–3 mm	41	18.98	10.53	10.53
3–4 mm	5	2.31	3.29	3.29
4–5 mm	3	1.39	3.29	3.29

Table 2.12

Total number and percentages of antral follicles of different sizes in pregnant groups

Follicle diameter	Early-mid pregnancy (*n* = 8; 391 sections)		Mid-late Pregnancy (*n* = 9; 457 sections)	
	Total number of follicles = 123	%	Total number of follicles = 147	%
< 2 mm	109	88.62	130	88.44
2–3 mm	13	10.57	17	11.56
3–4 mm	1	0.81	0	0.0
4–5 mm	0	0	0	0.0

2.4 Discussion

The cells of the OSE layer are the source of more than 90 percent of ovarian cancers. Pregnancy plays a crucial role in the prevention of ovarian cancer, but the mechanisms behind this role remain unclear. Understanding these mechanisms will increase the ability to develop effective prevention methods for those who are at risk of this disease. The following research focuses on the relationship between the OSE cells and growing follicles, and the changes that occur within the OSE layer during the different stages of reproductive status in the ovary. The results suggest that the protective role of pregnancy against EOC could be influenced by the reduction in regulation of follicular activity caused by pregnancy factors that lead to a reduction in the levels of follicular products (hormones and growth factors). Thus the results suggest that during pregnancy and anoestrus the proliferative activity of the OSE cells is suppressed and the rate of follicular growth is also reduced.

The immunohistochemical analyses of the OSE layer using PCNA and Ki-67 clearly revealed proliferative activity mostly in cycling ewes. Proliferating cells were detected only in close proximity to steroidogenic antral follicles and CL suggesting that ovulation stimulates multiplication of OSE cells. This may be a mechanism that enables the post-ovulatory replenishment of existing cells with new ones.

Histological analysis shows a variation in the morphology of OSE cells that correlated to ovulation activity and location in relation to the ovulation site. At the apex of the ovulatory follicle, the OSE cells appeared as flattened and dilated in shape. This morphological transformation could be a response of the OSE layer to the ovulation process. It is believed that prior to ovulation, many factors, such as gonadotrophins, growth factors, and steroids, are released at the ovulation site (Wong & Leung 2007). These factors may induce OSE cell proliferation in order to accommodate expansion in the surface area that occurs during follicular growth. OSE cells secrete proteolytic enzyme (urokinase-type plasminogen activator) that degrades the follicular wall of the ovulatory follicles at ovulation (Murdoch & McDonnel 2002); these enzymes also disrupt the underlying basement membrane and the connective tissue of the ovarian stroma aiding in follicular rupture and the release of the mature oocyte (Bjersing &

Cajander 1975). Post-ovulation, the OSE layer is repaired by rapid proliferation over the newly formed CL through a re-epithelialization process. Over the CL, OSE cells became cuboidal in shape, which could be a form of active proliferating cells. It has been reported in mice that the highest immunostaining for the PCNA antigen was detected at the ovulation site and was associated with cuboidal cells (Tan & Fleming 2004). Another *in vivo* study showed that cuboidal GCs have a high rate of proliferation (five-fold) as demonstrated by Ki-67 immunostaining compared to flattened GCs (Silva-Buttkus *et al.* 2008).

Distinct features in follicular development were observed in anoestrus and pregnant ewes when compared to cycling animals. Pregnancy did not affect the early development of follicles (primordial and transitory), but did inhibit the later growth stages (primary, preantral, and antral). During pregnancy, several factors, notably high steroidal hormones produced in the feto-placental unit and CL (al Gubory *et al.* 1994), may retard follicle development and suppress OSE proliferation. Consequently, large antral follicles were absent in this group. In agreement with our finding, it has been reported that the diameter of the largest antral follicles at the latest stage of pregnancy does not increase beyond 1.5 mm and most of these follicles are atretic (al-Gubory & Martinet 1986). After week seven of pregnancy, the human placenta produces a massive amount of progesterone that results in up to 10 fold increase in the maternal circulating levels of this hormone (Yen 1994). In pregnant ewes, the placenta is the main source of progesterone after day 50 of gestation (Al-Gubory *et al.* 1999), it produces 5 times as much progesterone as the ovary (Linzell & Heap 1968).

In vivo and *in vitro* studies suggested a suppressive role of progesterone on follicle development. Ovaries from twin pregnant ewes had fewer growing follicles than the ovaries of singleton pregnancy (Bartlewski *et al.* 1999). Exposure to high levels of placental progesterone may alter follicular development during pregnancy. It has been shown that, exogenous administration of progesterone reduces LH pulse and exerts an atretic effect on the bovine dominant follicle (Savio *et al.* 1993).

It was reported that the levels of FSH for cycling ewes are similar to the FSH concentration of anoestrus ewes (Bister & Paquay 1983). FSH is the most important hormone in controlling folliculogenesis; it is crucial for the later stages of follicle growth

(Turzillo & Fortune 1993), and it is considered a survival factor for antral follicles. Several studies have indicated that FSH activates GCs differentiation and proliferation, and has the ability to reduce the number of atretic follicles grown *in vitro* in rodents (Tonetta & diZerega 1989), cows (Hulshof *et al.* 1995), sheep (Newton *et al.* 1999), and humans (Roy & Treacy 1993).

By producing inhibitory factors, such as AMH that is present in GCs of all growing follicles (Durlinger *et al.* 2002), high numbers of mature follicles may prevent early follicle differentiation (as seen in the case of anoestrus sheep) as AMH is a crucial factor for initial follicle recruitment.

In pregnant and anoestrus ewes, negligible or low Ki-67 staining was observed in GCs in late differentiated follicles. PCNA staining was more intense than Ki-67 and the staining followed a similar pattern. In terms of the reliability of PCNA and Ki-67 as suitable nuclear proliferative markers, PCNA expression was observed even in the resting primordial follicles with ameiotic oocyte, whereas Ki-67 was expressed only in the proliferating follicles (at late growth stages and up-regulated in cycling ewes). These results agree with a previous study that reported the absence of Ki-67 and MCM2 (Minichromosome maintenance protein-2) antigens; immunostaining was completely absent in the cells of primordial and transitory follicles suggesting that these follicles are in a quiescent state (Silva-Buttkus *et al.* 2008). PCNA immunostaining was considered a useful method for counting ovarian follicles, because of its ability to mark all oocyte nuclei of ovarian follicles at different maturity stages including primordial follicles that were strongly labelled (Muskhelishvili *et al.* 2005). PCNA expression in primordial follicle oocytes is not an indicator of cell proliferation as these oocytes are arrested in the first meiosis (Hirshfield 1991); however, PCNA expression could be related to the role of PCNA in DNA repair mechanisms. Several studies suggested that PCNA can be used as a reliable marker for proliferation (Tomanek & Chronowska 2006), but Ki-67 seems to be a better marker as PCNA expression can be confused with DNA repair or other DNA synthetic activities not necessarily associated with cell division. This is in agreement with Scholzen & Gerdes (2000) who argue strongly against considering PCNA rather than Ki-67 as a reliable marker for proliferation.

In the rodent model, OSE cell differentiation was investigated in the presence of exogenously supplemented oestrogens and progesterone (Gotfredson & Murdoch 2007). The cells varied in types from simple squamous to stratified. Stratification of epithelial cells increased with oestradiol treatment, and was completely suppressed with progesterone. Stratified cells were also found to be highly proliferative and prone to neoplasia. Apparently, progesterone restores the epithelium of postovulatory ovaries to a resting or non-proliferative state. In the present study, though such OSE morphological differentiation was not discernable, oestradiol production in cycling antral follicles and CL might have induced proliferative activity in nearby OSE cells. The same explanation also holds true for observed proliferative activity in differentiating follicular GCs.

During pregnancy, progesterone seems to have suppressed these events. It has been suggested that high levels of progesterone during pregnancy is responsible for follicular development inhibition in heifers (Adams *et al.* 1992; Ginther *et al.* 1996).

It is proposed that oestrogen-progesterone antagonistic regulation of OSE proliferation near preovulatory follicle and CL may lead to neoplastic transformations, which may eventually initiate epithelial ovarian cancer unless such cells are constantly eliminated as they are in apoptosis. Ghahremani and colleagues (1999) suggested that the disruption of apoptosis and failure of eliminating those OSE cells lining inclusion cysts may enhance the formation of ovarian cancer. It has been shown that progestin oral contraceptives induce apoptosis in primate OSE cells (Hankinson *et al.* 1992; Siskind *et al.* 2000). Rodriguez *et al.* (1998) suggested that apoptosis induction may be a mechanism for the protective factor of combined oral contraceptives against epithelial ovarian cancer. An *in vitro* study on normal and malignant human OSE cells revealed that treatment with pregnancy equivalent doses of progesterone induces apoptosis through activation of Fas/Fas ligand pathway (Syed & Ho 2003).

The gonadotrophins, LH and FSH are the other hormones regulating OSE proliferation. Their place in the possible transformation into cancerous cells was studied in mice. Immature CD1 mice were treated with hormones to stimulate superovulation (Burdette *et al.* 2006). The OSE in proximity to antral follicles and CL proliferated significantly more in superovulated animals than in controls. Proliferation was thought to be an act of healing surface wounds caused by ovarian surface rupture after each cycle

of ovulation. Initially, it was believed that free radicals and other mitogenic factors, such as oestrogens, generated at the rupture sites induced ovarian neoplasia. However, it was later observed that proliferation occurred before ovulation (Gaytan *et al.* 2005). This challenged the rupture and repair theory of proliferation. According to a recent gonadotrophin theory, the expression of LH and FSH receptors on the surface of OSE cells is a direct response to gonadotrophins (Leung & Choi 2007). Most likely, the hormone-stimulated receptors trigger the localized proliferation events.

In conclusion, the results indicate that sheep pregnancy leads to inhibition of proliferative activity in the OSE and suppression of follicular growth. Progesterone appears to be a candidate for suppressing OSE proliferation and preventing follicular differentiation, and thereby not allowing antral follicles to form. The effect of progesterone during pregnancy seems to override the positive effect of oestrogens on OSE proliferation and follicle development. Conditions that suppress ovulation, such as multiple pregnancies, breastfeeding, and the use of oral contraceptives, suppress follicular differentiation and OSE proliferative activities. This seems to be due to high serum progesterone and low LH and FSH concentrations. High serum progesterone may have a direct negative effect on gonadotrophin production and might inhibit events leading to both follicular development and OSE proliferation.

Our knowledge of the interaction between preovulatory follicles and the OSE layer is not yet fully elucidated**,** and further *in vitro* study is needed to definitively determine the effect of steroids and other factors involved in ovulation on the proliferative activity of OSE.

Figure 2.11 Box plots indicating the percentage of PCNA and Ki-67 immunoreactivity in GCs of growing follicles. (A) PCNA and (B) Ki-67 immunoreactivity in GCs of primary, preantral, and antral follicles at cycling (n = 6; 18 sections), anoestrous (n = 5; 15 sections), early-mid (E-M) pregnant (n = 8; 24 sections), and mid-late (M-L) pregnant (n = 9; 27 sections) groups. The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Values are percentages of labelled GCs per follicle, and different letters above the bars indicate statistical differences between groups ($P \leq 0.05$). The total number of analyzed follicles is outlined in Table 2.5 and 2.6.

Figure 2.12 Box plots representing the distribution of ovarian follicles at different reproductive status. The distribution of (A) primordial, transitory, and primary follicles and (B) preantral and antral follicles at cycling (n = 6), anoestrous (n = 5), early-mid (E-M) pregnancy (30–75 days) (n = 8), and mid-late (M-L) pregnancy (80–135 days) (n = 9). Lines on the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Values are percentages of each stage of development per animal, and different letters above the bars indicate statistical differences between groups ($P \leq 0.05$). The total number of analyzed follicles is outlined in Table 2.7 and 2.8.

CHAPTER THREE

In Vitro Regulation of OSE Proliferation by Local Ovarian Factors

3.1 Introduction

The previous chapter looked at OSE proliferation *in vivo* and showed how it was related to proximity to other ovarian structures and how this effect is generally due to specific endocrine/ paracrine factors. However, to investigate how specific growth factors and hormones affect proliferation it is necessary to use an *in vitro* model. Obtaining OSE cells for culture has been problematic because of its fragile and tenuous attachment to the underlying layers, and the limited ability of OSE cells to grow in cultures.

The first culture systems for OSE were achieved in the 1980s for rats (Adams & Auersperg 1981), humans (Auersperg *et al.* 1984) and rabbits (Nicosia *et al.* 1989). The principal method for OSE isolation is a gentle scraping of the ovarian surface, as the OSE cells adhere only loosely to the underling stroma layer. Extracellular calcium is a controller for OSE proliferation, which is mediated by calcium-sensing receptors (McNeil *et al.* 1998). Therefore, every culture medium depends on a certain level of calcium. Usually OSE cells in cultures do not proliferate more than four passages before they transform into large, senescent flat cells. There are differences in the physiology and morphology of the OSE between species, which is likely to be related to changes in the reproductive status of the species (Auersperg *et al.* 2001).

Normal OSE cells express receptors for several growth factors such as keratinocyte growth factor (KGF), EGF, HGF, TGF-ß (Berchuck *et al.* 1991; 1992; Parrott *et al.* 2000b). Additionally, oestrogen, androgen, and progesterone receptors are expressed in normal OSE cells in humans (Karlan *et al.* 1995) and rats (Adams & Auersperg 1981). Several studies have shown that steroids increase OSE proliferation *in vitro,* in a dose-dependent manner (Wright *et al.* 2005). LH and FSH receptors are present in bovine (Parrott *et al.* 2001) and human OSE cells (Syed *et al.* 2001). There are indications that LH and FSH have the ability to stimulate OSE cell proliferation in human and ovine OSE cells (Gubbay *et al.* 2004). Intra-ovarian factors play an important role in modulating OSE function, and they might be involved in the malignant transformation of these cells (Parrott *et al.* 2001).

Follicular fluid (FF) is a major constituent of the mature ovarian follicle; it plays an important role in most of the ovarian physiological processes. Steroidogenesis, oocyte maturation and ovulation depend on the endocrinological alterations in the FF. The ovarian fluid is made up of a different novel secretion e.g. steroids hormones, peptides and glycosaminoglycans (GAGs), and a plasma exudate, especially its proteins. The concentration of hormones within the FF changes in correlation to the stage of follicular development. The concentration of steroid hormones in human FF was extensively reviewed by Lenton & colleagues (1988). There are several studies determining steroid contents in FF in different species, specifically after the LH surge (Chaffin *et al*. 1999; Dieleman *et al.* 1983). According to Baird & Fraser (1975), the level of steroid hormones in FF differs markedly approaching ovulation. A noticeable shift from oestrogen to progesterone production is a major event in the preovulatory phase (Hillier 1985). In the human, after the LH surge, progesterone concentration reaches approximately 17500ng/ml follicular fluid, whereas oestrogen concentration declines from 3700 ng/ml before the LH surge to 800ng/ml after 30 hours of the LH surge (Lenton *et al*. 1988). Microarray for gene analysis revealed that the expression of StAR, CYP17A1 and P450 Oxidoreductase were increased in preovulatory follicles (Agca *et al*. 2006). Gonadotrophins, FSH and LH are present in FF and their concentrations increase in preovulatory follicles, although remaining below levels in systemic blood.

Rodents have been used in several studies investigating the function and physiological roles of normal OSE (Burdette *et al.* 2006; Gaytan *et al.* 2005); however, ruminant models with reproductive cycles relatively similar to the human cycle are preferable. Sheep OSE have been shown to be an adequate model for *in vitro* studies on human OSE (Gubbay *et al.* 2004) which is why this study uses them as a model.

Cytokeratins (CKs) belong to the intermediate filaments, which create a cytoskeleton in almost all eukaryotic cells. The CK family is a highly complex multi-gene family of polypeptides, the molecular weight of which ranges from 40 to 68 kDa. CKs are generally held to belong to the most fundamental markers of epithelial differentiation, and until now, 20 distinct CK polypeptides have been found in various human epithelia (Moll 1998). OSE is characterized by keratin types 7, 8, 18 and 19,

which represent the keratin complement typical for simple epithelia (Auersperg *et al.* 2001).

In the study in Chapter 2, a sheep model was used to determine the role of local ovarian structures (growing follicles and corpora lutea) in the regulation of OSE proliferative activity. The aims of the following study are to examine the effects of follicular and luteal products on the proliferation of OSE cells in culture, and to analyse the influences of large antral follicles and corpora lutea on the expression of gonadotrophin receptors by the OSE.

3.2 Materials and methods

3.2.1 OSE isolation and cell culture

Sheep ovaries were collected from adult cycling sheep immediately after slaughter at a local abattoir. Ovaries were transported to the laboratory in a sterile thermos containing culture media of M199/MCDB 105 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma).

OSE cells were obtained by gently scraping the surface of the ovaries (n = 20) using the blunt edge of a sterile scalpel. OSE cells were transferred into sterile flasks (n = 4), which were pre-coated with foetal calf serum (FCS), and contained culture media of M199/MCDB 105 (1:1) supplemented with 1mM L-glutamine, 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. OSE cells were examined with a phase-contrast microscope to ensure sufficient flakes of OSE had been obtained. OSE cells were incubated at 37 ºC in a humidified incubator with an atmosphere of 5% CO2 in air for up to 28 days with media changed every 7 days according to Hillier *et al.* (1998). Confluent cells were normally obtained after three to four weeks.

3.2.2 Cytokeratin immunocytochemistry (ICC)

To confirm the purity of the cultured cells (epithelial origin), a fraction of the cells were seeded in chamber slides (Sigma-Aldrich Ltd) for ICC to detect cytokeratin (a marker of epithelial cells). Cells were fixed in ice-cold 80% methanol, air-dried then washed in PBS, followed by incubation for 20 min with 10% normal horse serum and further 30 min with cytokeratin antibody (1:50 mouse monoclonal cytokeratin Clone MNF116 Dako Cytomation, Denmark A/S) at room temperature. Following the washing stages, cells were incubated for 30 min with anti-mouse IgG Biotinylated antibody. Finally, cells were incubated with Vectastain Elite ABC kit (Vector Laboratories, Inc. California, USA) for 30 min then washed in PBS. For visualization, slides were incubated with DAB for 10 min, then counterstained with haematoxylin.

3.2.3 Proliferation assay

In order to investigate the effect of steroids and growth factors on OSE cell proliferation, confluent cells were recovered using trypsin EDTA (0.05% trypsin and 0.02% EDTA) for 5 min at 37 ºC. Fresh media was added to the trypsinised cell pellet and a fraction was taken to be counted using a haemocytometer.

OSE cells were seeded into 96 well plates at a density of 5,000 cells per well and cultured for 24 h in media containing serum to ensure cell attachment. Cells were incubated for a further 24 h in serum-free media containing 0.01% bovine serum albumin (BSA). The treatments (each treatment was applied to three different wells) were administrated to the cells and incubated for 72 h.

Follicular fluid was aspirated from medium (6–8 mm, n = 20) and large (10–12 mm, n = 15) bovine antral follicles from the ovaries (n = 18) obtained from a local abattoir. A fraction of the fluid was charcoal-extracted to achieve a steroid-free fluid (van Tol & Bevers 2001). Extracts from mature bovine corpora lutea (n = 7), progesterone (3 ng/ml, 30 ng/ml and 3,000 ng/ml; Sigma-Aldrich), 17β-oestradiol (3 ng/ml, 30 ng/ml, 3,000 ng/ml; Sigma-Aldrich), recombinant human IGF-1 (100 ng/ml; NHPP, Torrance, California), and FCS (final concentration in culture media, 10%) were used as a positive control for the assay. Luteal extracts were obtained by grinding freshly collected bovine corpora lutea. Ground tissue was filtered, centrifuged and sterilized using sterilization filter, and then stored at –20 ºC. Doses of steroids were representative of preovulatory follicular fluid levels (reaches levels of 1 µg/ml E_2 and 17 µg/ml P_4) and circulating levels (range 1 ng/ml E_2 and 30 ng/ml P_4) (Lenton *et al*. 1988).

OSE cell proliferation was measured using the Cell Titer 96® AqueousOne Solution Proliferation Assay (Promega, Southampton, UK) which is based on the conversion of MTS tetrazolium to a coloured formazan product by viable active cells. Following the instructions of the manufacturer, 20 µl of the MTS/PES solution was added to each well, followed by three hours incubation in a humidified atmosphere. The amount of formazan converted during this period was measured as absorbance at 490 nm in a spectrophotometer. Cell growth in untreated control cells was assigned a value of 1, and relative cell growth in a treated culture was expressed as fold change over the

untreated control cells. Data points are group mean values ± SEM from four independent experiments. For each treatment, mean values were calculated from triplicate wells.

3.2.4 Real-time PCR for expression of LH-R and FSH-R

To perform real time reverse transcriptase polymerase chain reaction, OSE cells were obtained by scraping the surface of ovaries from cycling sheep (n = 20). Cells were collected from different areas of the ovary: areas overlying large antral follicles (≥ 5 mm; n = 17), areas covering mature corpora lutea (n = 12), and areas over the ovarian stroma (i.e., no visible underlying follicles or CL). Six different pools of cells corresponding to each of the three areas (2 different pools for each area) were obtained from 20 animals, and RNA was immediately extracted.

The Tri Reagent (Sigma-Aldrich) method was used to extract the total RNA from suspended ovarian surface epithelial cells. Following the manufacturer's instructions, RNA samples were purified by a phenol-chloroform extraction followed by precipitation with ethanol. The amount of RNA was estimated by spectrophotometer at 260/280 nm. One microgram of each total RNA was reverse-transcribed using Super Script III Reverse Transcriptase (Invitrogen Corp., Carlsbad), and using random primers (Promega, Madison, WI, USA) and a dNTP mix to generate single-strand cDNA for each sample using the thermocycler (Biomefra TGradient). Selection of primers (Table 1) for LHR, FSHR, and 18S was performed using LightCycler Probe Design software (Roche Applied Science, Mannheim, Germany). Quantification of mRNA levels for LHR and FSHR was analysed using a RT-PCR cycler (Stratagene Mx3000P). A portion (1/4) of each cDNA was used for quantitative PCR using a master mix (2X SensiMix DNA kit, Quantace Ltd., London, UK) that included SYBR green solution, SensiMix, and $MgCl_2$. PCR settings were in all cases set at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 30 sec. All real-time experiments were run in triplicate, and mean values were used for the determination of mRNA levels. The negative controls consisted of RT-negative (RNA template with no reverse transcriptase enzyme) and RT-H_2O (water in place of RNA template). The abundance of each target mRNA was determined relative to the housekeeping gene 18S. Changes in

the average fold was calculated with Mx3000P real-time PCR system analysis software (Stratagene) using the cycle threshold for each sample relative to a standard curve constructed from RNA extracted from granulosa cells.

Table 3.1

Primers for real-time PCR

Gene	Forward primer	Reverse primer	Accession number	Product size
oLHR	5'-TCTTTGCTGAGAGTGAACTGAGTGA-3'	5'-CGGGAGCACATTGGAGTGT-3'	L36329.1	78 bp
oFSHR	5'-CAACCCCTTCCTCTACG	'5-GGAACCATTGGTAACCCT-3'	NM_001009289	184 bp
18S	'5-GGGGAATCAGGGTTCG-3'	5'-GCTGGCACCAGACTTG-3'	AJ311673	209 bp

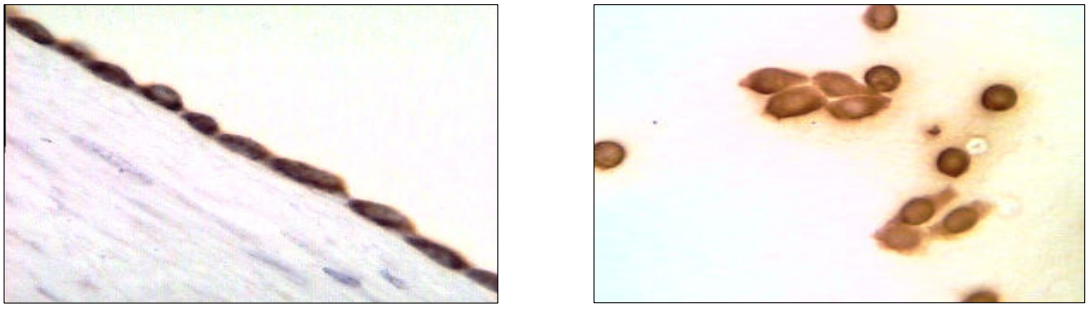
3.2.5 Statistical analysis

The Kolmogorov-Smirnov test and Box plot graphs were used in order to decide if the data was normally distributed. Data for proliferative response of OSE cells to different treatments was presented as mean values from four independent experiments. Results were normally distributed, therefore one-way analysis of variance (ANOVA) was used as a robust test for analysing normally distributed data. After a significant ANOVA, a Tukey post-hoc test was performed in order to locate the significant differences between the groups. Analysis was done using Minitab version 15. Data for gene expression was presented as mean values from three different assays, data was not normally distributed in accordance to the Kolmogorov-Smirnov test; therefore, the Kruskal-Wallis test was selected because it is the non-parametric analogue of ANOVA that compares more than two groups. The Dunn's test was used as a post-hoc test to locate the significant differences between the groups. Analysis was conducted using GraphPad Prism statistical software. Differences were considered to be significant at $P \leq 0.05$.

3.3 Results

3.3.1 Cytokeratin expression in OSE cells

To confirm the purity of the epithelial cells in the ovine isolated cells, immunohistochemistry against cytokeratin was performed. Cultured OSE cells stained positively for keratin. Also, the complete intact layer of the OSE was completely stained with the antibody. No negative cells for cytokeratin immunostaining demonstrating the purity of the culture (Figure 3.1).



A **B**

Figure 3.1 Cytokeratin expression in ovine OSE. Immunohistochemistry for (A) intact OSE cells (*in vivo*) and (B) isolated OSE cells (*in vitro*), at 600X magnification.

3.3.2 Proliferative response of cultured OSE cells to follicular fluid and CL extracts

The effects of the main ovarian compartments of follicular fluid and CL on the proliferative activity of the ovine OSE cells were investigated. Cells were grown in the presence of the treatments or medium alone (control). Administration of FCS was used as a positive control for the proliferation assay. Incubation with follicular fluid and CL extracts stimulated the proliferation of cultured OSE an average of about 2.5 fold relative to the control (untreated cells) (P= 0.001). There were no effects of follicle size or previous charcoal extraction in the proliferative response to follicular fluid (P= 0.1) (Figure 3.2).

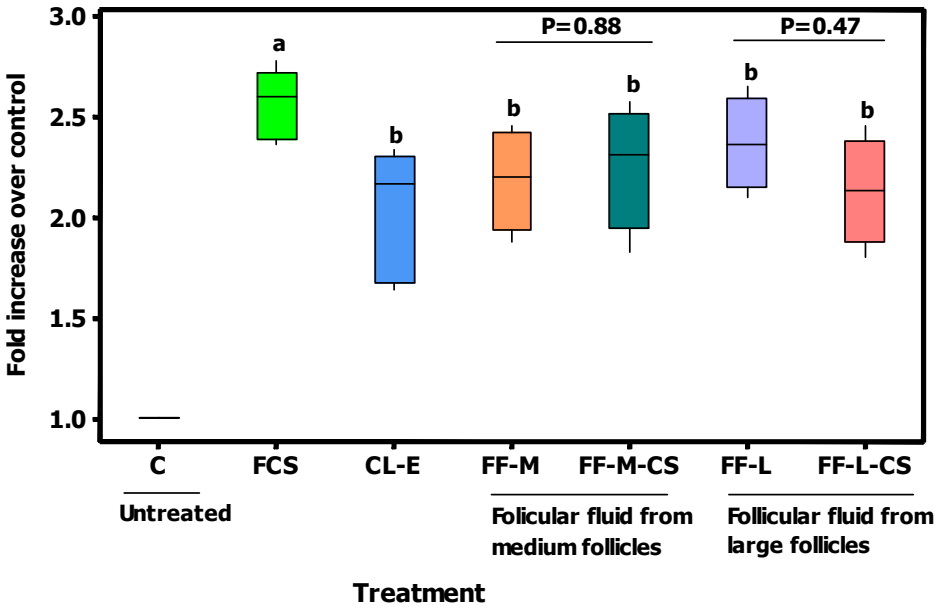


Figure 3.2 Box plots are of the proliferative responses of cultured sheep OSE cells to FCS, corpora lutea extracts (CL-E, *n* = 7), follicular fluid from medium follicles (6–8 mm) (FF-MF, *n* = 20) and large follicles (10–12 mm) (FF-LF, *n* = 15). Follicular fluid was separately pooled from medium or large follicles, and a fraction from each pool was charcoal-stripped (CS). Each box plot represents the means of four independent assays for each treatment, and lines represent the whiskers. Values are expressed relative to the value in untreated cells (control) that was taken as 1. Different letters above bars indicate a statistical difference between each treated group and the control, P≤ 0.05.

3.3.3 Effect of steroid hormones and growth factors on the proliferation activity of cultured ovine OSE

Cultured OSE cells were treated with progesterone or oestrogen in a number of concentrations. Steroids administration at different doses (low and high) had no significant effect on cell proliferation (P= 0.1). Only IGF-1 administration induced OSE cells proliferation (mean of 2.2 fold over control; P= 0.01) (Figure 3.3).

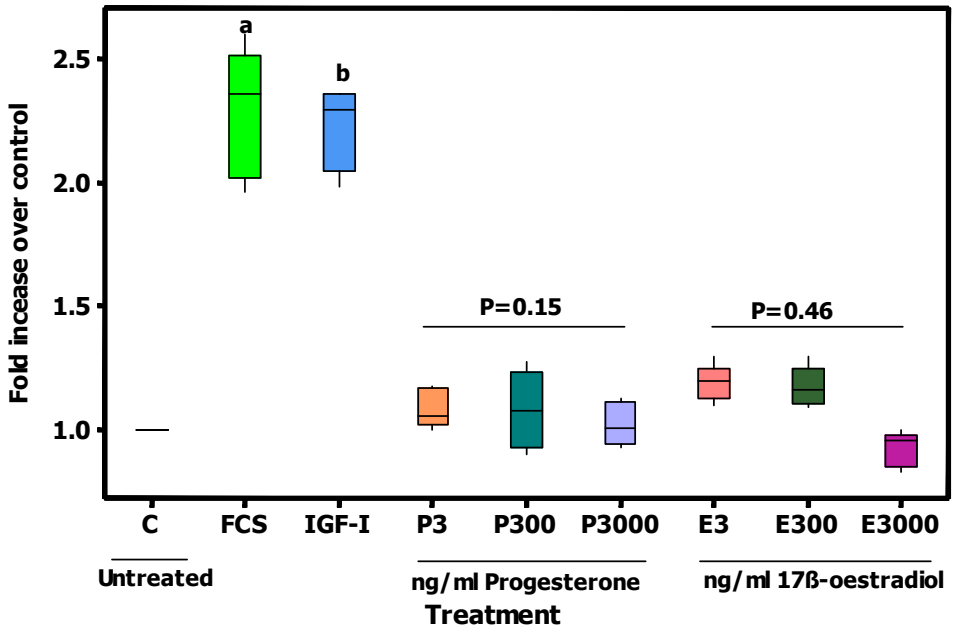


Figure 3.3 Box plots are of the proliferative responses of cultured sheep OSE cells to FCS, recombinant human IGF-1 (100 ng/ml), progesterone (P) (3,300 and 3,000 ng/ml) and 17β-estradiol (E) (3,300 and 3,000 ng/ml). Each box plot represents the mean of four independent assays for each treatment, and lines represent the whiskers. Values are expressed relative to the value in untreated cells (control) that was taken as 1. Different letters above bars indicate a statistical difference between each treated group and the control, $P \leq 0.05$.

3.3.4 FSHR and LHR expression

To investigate whether gene expression of FSHR and LHR were regulated by the underlying ovarian compartments, the levels of FSHR and LHR mRNA in the OSE cells (isolated from different areas around the ovary) were determined. Both receptors LHR and FSHR showed a variation in the level of their expression. Distribution of FSHR was statistically varied between the three areas. The highest expression was detected in the OSE cells collected from the area over the large antral follicles ($P \leq 0.01$). In OSE cells overlying the ovarian stroma, mRNA of FSHR significantly decreased ($P \leq 0.01$). OSE cells over the CL expressed very low levels of FSHR (Figure 3.4). Although the level of LHR expression was not significantly different among the three areas, the distribution of the receptor was following the same trend of the FSHR with the highest level of LHR expression was reported in OSE cells isolated from the area over the large follicles.

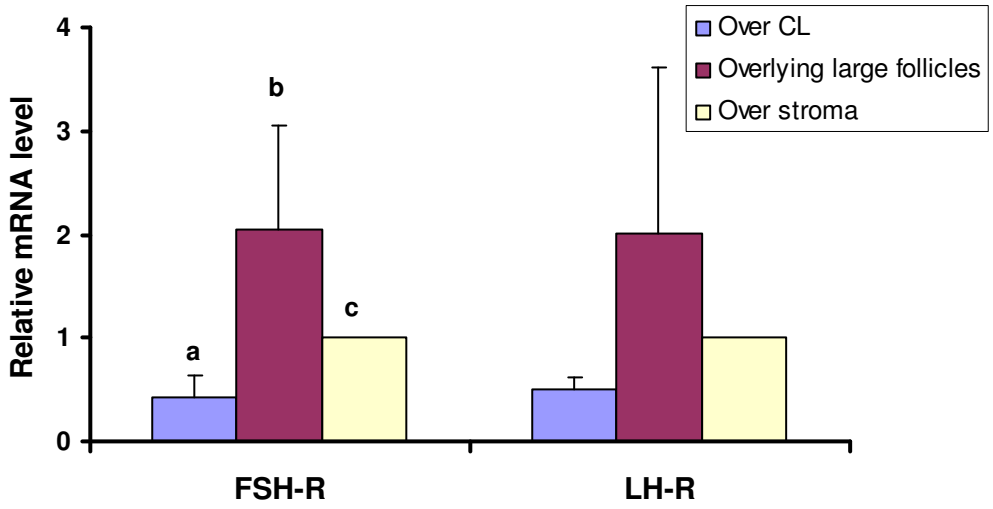


Figure 3.4 Expression levels of LHR and FSHR in ovine OSE cells over mature corpora lutea (CL) (n = 12), over large antral follicles (n = 17), and over stroma. Data were standardized to the stroma means, and are presented as means ± SEM of three independent assays. Different letters above bars indicate a statistical difference between the three different areas, $P \leq 0.05$.

3.4 Discussion

During every cycle, the OSE over the preovulatory follicles is exposed to high concentrations of steroids, growth factors, and inflammatory reagents. There is evidence that OSE cells proliferate after ovulation in order to repair the ruptured area. Preovulatory follicles are the main source of several hormones and growth factors, and these factors are implicated in the tumourgenesis of the OSE cells through mitogenic effects. The current study investigates the effects of steroid hormones produced in a high concentration by the antral follicles on the proliferative activity of the OSE cells. The results show that factors in follicular fluid can induce OSE proliferative activity, and this stimulation effect could be not attributed to steroids in follicular fluid since oestrogen and progesterone treatments failed to stimulate OSE cells. Other factors within the follicular fluid are responsible for the mitogenic effect of this fluid.

The addition of FCS in the serum-free medium enhanced OSE cell proliferation when compared to controls. FCS seems to contain some undefined compound(s) that stimulates proliferation. Serum supplementation provided essential nutrients (amino acids, vitamins, lipids) and adherence material for growing cells (Masters 2000). Reports suggest that some undefined serum factors may influence epithelial-mesenchymal cell transformation (Auersperg *et al.* 2001). Fetuin, a glycoprotein component of FCS that contributes to attachment of the cultured cells, may cause adherence of the epithelial cells in a way that happens in re-epithelialisation of the OSE layer. It has been found that Fetuin-A, a Ca^{2+}-dependent adhesion factor in FCS induces cell-to-cell adhesion leading to tumorigenesis (Kundranda *et al.* 2005). Initial adherence may influence synthesis of cellular Ca^{2+}-dependent adhesion factors, such as N- and E-cadherins, and these in turn may influence proliferative activity.

In this study, follicular fluids from medium and large follicles and extracts of CL stimulated the growth of OSE cells. Follicular fluid is partially an exudate of serum, and therefore it is expected to contain at least some of the mitogenic factors found in serum.

These extracts or fluids contained a number of anterior pituitary glycopeptide hormones, FSH and LH/hGC, steroidal hormones, oestrogen and progesterone, and several localized growth factors, such as IGF and HGF. In addition, receptors of these

follicular modulating hormones are present on the surface of OSE cells. While most of the steroidal hormones regulate follicular cycle and maintain luteal cycle, their role in the OSE is not clearly understood. Oestrogens are implicated in OSE neoplastic transformation. In order to examine whether steroidal hormones affect OSE proliferation, the follicular fluids and CL extracts were processed by passing them through charcoal to remove steroidal components. However, even these steroid-free fluids accelerated OSE proliferation over the serum-free controls. Apparently, steroids have no direct effect on *in vitro* OSE cell multiplication. Steroids may regulate the growth of OSE cells via indirect effects. It has been demonstrated that oestrogen administration induces ovarian cancer cell proliferation by increasing the expression of TGF-α (Simpson *et al.* 1998). Previous investigation has indicated mitogenic effects of TGF-α and EGF on bovine OSE cells (Doraiswamy *et al.* 2000). In order to further verify the ineffectiveness of steroidal hormones, pure oestrogen (oestradiol) and progesterone at graded concentrations were added to the cultured OSE cells. Neither hormone made any significant difference in proliferative activity of OSE cells over the controls, which is consistent with observations of the human and rhesus OSE cells (Ivarsson *et al.* 2001; Wright *et al.* 2003). The results of the current study suggest that factors other than steroids may be responsible for *in vitro* proliferation of OSE. The likely candidates could be gonadotrophins and the local growth factors produced in follicles, which trigger a sequence of reactions leading to proliferative response.

FSH in particular has been implicated in increasing *in vivo* OSE proliferation (Choi *et al.* 2007). However, *in vitro* results were conflicting, and in various species stimulatory, inhibitory, and non-responsive influences of these hormones were noticed. One explanation for such variation is that the differential expression of surface gonadotrophin receptors and follow-up downstream signalling molecules affect proliferation. It has been reported that hCG and oestradiol may regulate OSE proliferation indirectly through an IGF-I pathway (Wimalasena *et al.* 1993). In addition, a study in bovine OSE revealed that hCG and FSH could increase the levels of mRNA of KGF and HGF (Shoham 1994). These growth factors utilize multiple signal pathways to suppress OSE apoptosis, and thus may lead to a mitogenic effect resulting in OSE malignancy. It was a matter of interest to examine whether such localized growth factors

have a direct influence in the multiplication of ewe OSE cells. It was observed that relative to controls IGF-1 stimulated the proliferative response over two times. It can be presumed that in cycling ewes the oestrogen response to OSE proliferation could be mediated in part by an increase in the transcription of IGF-1 and HGF. Charcoal stripping is a useful method to eliminate steroids, but also may remove cytokines and growth factors from serum (Mckeehan *et al.* 1984). However, in this study we suggest that charcoal stripping did not remove all growth factors from FF and IGF-I may be the factor responsible for the stimulatory effect of FF on OSE cell proliferation.

FSH and LH may also mediate through their receptor expression (an independent transcriptional activation of such growth factors), and thus the ultimate manifestation of oestradiol and gonadotrophins is the same. One of the underlying signal transduction operating transcriptional controls is the cAMP-dependent protein kinase A (PKA)-mediated phosphorylation (Gubbay *et al.* 2006). One factor is the cAMP response element binding protein (CREB) and the activating transcriptional factor-1 (ATF-1). In sheep, CREB/ATF-1 accounted for the survival of OSE through the stimulation of proliferative activity, and the prevention of apoptosis. OSE cells are exposed to the gonadotrophin inflammatory response during ovulation, and cytokines e.g. interleukins-1, -6 may induce oncogenic response. Elimination of such cells by apoptosis at the rupture site during post-ovulatory epithelialisation is presumably an oncoprotective mechanism. In the surrounding regions, gonadotrophins stimulate an opposite response by evading apoptosis and up-regulating CREB/ATF-1 expression. Thus collateral OSE cells take over the rupture site and contribute to the healing process. In humans, the gonadotrophin cAMP-PKA signalling pathway was found to down-regulate N-cadherin protein (Pon *et al.* 2005). N-cadherin, a Ca^{2+}-dependent cell-to-cell adhesion protein, was found to be controlling the survival capabilities of OSE. In the absence of adhesion due to N-cadherin down-regulation, the cells were non-aggregated and had a tendency to undergo apoptosis. This feature is observed at the postovulatory rupture site. Thus follicular hormones appear to have both antagonistic effects on OSE cells at the rupture site and in the surrounding region.

Although the expression of gonadotrophin receptors in the OSE has been reported in several species (Parrott *et al.* 2001; Syed *et al.* 2001), the distribution of

receptor expression across the ovarian surface has not previously been considered. The expression of LH and FSH receptors over large follicles (5 mm or larger) were two and four times higher than those over stroma and CL respectively, suggesting that OSE proliferation over growing follicles results at least in part from a local increase in the sensitivity of OSE cells to circulating gonadotrophins. It has been reported that treatment of anoestrous sheep with oestradiol increased LH receptor expression in the OSE (Murdoch *et al.* 1999), and in granulosa cells both oestradiol and IGF-1 up-regulate the expression of gonadotrophin receptors (Knecht *et al.* 1984; Hirakawa *et al.* 1999). Therefore, an increase in gonadotrophin receptor expression in the OSE overlying large follicles may be mediated by paracrine follicular influences.

In conclusion, OSE proliferation in cycling sheep is associated with underlying developing follicles and CL, mediated by, at least in part, the up-regulation of gonadotrophin receptors, and facilitated by the action of mitogenic glycopeptides and growth factors, but not steroids.

The last two chapters focused on the effect of the mitogenic factors within the ovary and their roles in OSE regulation. The study would be more inclusive if the role of apoptosis in the physiology of the OSE layer was investigated. It might be that apoptosis in pregnancy is a protective factor for ovarian cancer. Further, changing the model system to an animal with more similarities to humans (no seasonality) would improve the conditions of the study.

CHAPTER FOUR

In Vivo and *in Vitro* Studies on Apoptosis in OSE Cells and Inclusion Cysts of Pregnant Heifers

4.1 Introduction

The OSE shows a high degree of metaplasia transformation to a differentiated state, and this can lead to the development of EOC. The first step in tumourigenesis of the surface epithelium is the formation of epithelial inclusion cysts derived from crypts or invaginations of the OSE (Cramer & Welch 1983). During post-ovulatory repair, OSE cells tend to modulate to fibroblast-like mesenchymal cells with an appearance similar to the underlying stroma. The epithelio-mesenchymal conversion of the OSE layer is considered to be a homeostatic mechanism that helps to accommodate trapped OSE cells after ovulation, and then to incorporate them into the stroma as part of stromal fibroblast (Auersperg *et al.* 2001). Some displaced segments of damaged OSE fragments retain the epithelial features and produce surface invaginations (cleft) and inclusion cysts in the ovarian cortex (Murdoch 1994). The healing process also results in the formation of crypts in the ovarian surface, which penetrates the ovarian stroma, where they form inclusion cysts lined with OSE cells (Godwin *et al.* 1993).

Why these otherwise non-proliferating resting OSE cells become tumourigenic has been explained through the "incessant ovulation theory" (Fathalla 1971), which speculates that repeated ovulation leads to malignancy due to frequent rupture and repair of the damaged OSE layer. Under the influence of mitogens and other stress factors, some of the mutagenized cells undergo neoplasia transformation (Wong *et al.* 2004). Women with multiple pregnancies or those who are on oral contraceptives have shown reduced risks of EOC (Risch 1998; Ness *et al.* 2000). The validity of Fathalla (1971) hypothesis has been challenged because progestin-only contraceptives, which do not restrict ovulation, have been found to be as effective in suppressing malignancy as the oral ovulation-inhibitory contraceptives. Moreover, women with polycystic ovarian syndrome who have decreased ovulatory cycles have a high vulnerability towards ovarian cancer (Spritzer *et al.* 2005). Risch (1998) suggests that the protection gained from pregnancy is through the 8-9 months of continuously high progesterone levels. *In vitro* and *in vivo* analyses have shown that progesterone inhibits regular cell cycle by inducing apoptosis in normal and malignant human OSE cells (Bu *et al.* 1997; Hu & Deng 2000). The domestic chicken (hen) is a persistent ovulating animal, and like

humans it also develops ovarian cancer which reacts with a panel of antibodies specific for human ovarian cancers (Giles *et al.* 2006)Rodriguez-Burford *et al.* 2001). Hens treated with progesterone have shown decreased incidences of EOC, supporting the hypothesis that progesterone induces apoptosis of damaged OSE cells (Fredrickson 1987; Rodriguez *et al.* 1998). Progesterone has also been shown to reduce the risk of developing ovarian carcinoma in postmenopausal women who have undergone oestrogen and progesterone replacement therapy (Schneider & Birkhauser 1995). Such therapies have also clinically helped treatment of selected ovarian tumours (Key 1995). Administration of progesterone at a high concentration similar to levels during pregnancy inhibited cultured human OSE cell proliferation (Syed *et al.* 2001). There is also growing evidence indicating an etiological role of localized inflammation, which accompanies each ovulation, with an associated release of cytokines, mitogens and intrusion of inflammatory cells, leading to genetic damage in the OSE cells (Landen, Jr. *et al.* 2008). It has been proposed that progesterone most likely has a role in apoptotic elimination of such genetically damaged OSE cells derived from inflammatory and mitogenic responses, although the underlying mechanism is not fully understood.

Apoptosis in a tissue is manifest as distinct morphological characteristics and energy dependent biochemical changes (Elmore 2007), that helps in normal cell turnover, development and functioning of the immune system, hormone dependent atrophy, embryonic development, chemical induced cell death etc. Aberrations in apoptosis lead to neurodegenerative diseases, ischemic conditions, autoimmune diseases and several forms of cancers, including EOC (Baldwin *et al.* 1999). Microscopically, cell shrinkage and pyknosis, condensation of cell organelles and chromatin, cell clustering and excessive plasma membrane blebbing are observable. The fragmented cells are eventually phagocytosed by macrophages. Three interlinked biochemical processes (the extrinsic, intrinsic and granzyme B pathways) execute a common terminal pathway in which caspase 3 (cysteinyl aspartic acid protease 3) is activated and that in turn triggers DNA breakdown, protein cleavage and cross-linking, and surface phagocytic recognition. Caspases are widely expressed in all kind of cells as inactive pro-enzymes, and once activated start to proteolytically activate the other pro-caspases in a cascade of reactions. To date ten different caspases have been recognized and

categorized as the initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7) and inflammatory caspases (caspase 1, 4, 5). Caspase-3 expression and activation has been detected in the ovary of rats (Boone & Tsang 1998), mice and human (Matikainen *et al.* 2001) and hens (Johnson & Bridgham 2000). In mice, deletion of the caspase -3 gene results in apoptosis failure in granulosa cell (Matikainen *et al.* 2001) and aberrant luteal regression (Carambula *et al*. 2002). Moreover, inactivation of caspase-2 gene significantly decreases germ cell death and consequently leads to an elevation in the number of primordial follicles in mice ovaries (Bergeron *et al*. 1998).

Mechanistically, the mitochondrial transmembrane permeability and electrical gradient is altered causing release of cytochrome c, the Second Mitochondria derived Activator of Caspase (Smac)/DIABLO and other proteins, that complex with procaspase-9 to form "apoptosome" for final activation of capsase 3. Members of B-cell leukaemia/lymphoma 2 (Bcl-2) family of proteins activated by tumour suppressor protein p53 is responsible for maintaining mitochondrial permeability and consequently promoting apoptosis. Depleted growth factors, hormones, cytokines, and radiation, reactive oxygen species (ROS), toxins, hypoxia etc. can initiate intrinsic apoptosis.

A key component in investigating apoptosis is P53, a tumour suppressor protein, also referred to as "guardian of the genome", is an important regulator of cell cycle by blocking progression through G_1 phase (Livneh & Fishman 1997). Either it triggers apoptotic response or halts the cell cycle, including cyclin-dependent kinase inhibitor p21. Both p53 and p21 were also suggested to regulate G_2/M checkpoint that is the transition from G_2 to M phase. Mutations in p53 or inactivation through interaction with viral or cellular proteins are the most frequent alterations observed in cancer cells (Levine 1997). *P53* gene was found to be over-expressed as a result of repetition of ovulation trauma each cycle (Aunoble *et al.* 2000).

It is unclear whether steroidal hormones, specifically progesterone, suppress OSE malignancy by activating tumour suppressor genes, in particular *p53*, and direct the cells towards apoptotic destruction from the ovulation site. A connection between progesterone and p53 was evident from the work on epithelial layer of salivary glands. Adenoid cystic carcinoma in these cells was accompanied with increase in surface progesterone and oestrogen receptors and concomitant higher expression of p53 (Barrera

et al. 2008). Since OSE cells also express p53 besides oestrogen and progesterone receptors, and are exposed to changing levels of steroid hormones, this study hypothesized that during pregnancy an accumulation of progesterone may be a factor responsible for p53-mediated apoptotic progression of mitogenic and mutationally damaged OSE cells.

In this section of the study we used the cow as an animal model since this is a mono-ovular animal that ovulates in a regular pattern, has the same size of follicles (~ 20 mm) and luteal phase similar in length to that of the human (~ 15 days), although the follicular phase is shorter in heifers (2-3 days). Even though these animals spend their short life span either gestating or lactating, bovine ovarian cancer has also been reported (Marchant 1980), suggesting that the tumourigenic potential of bovine OSE is similar to human OSE. Therefore, bovine ovaries present a useful model for studying oncology of OSE and inclusion cysts. Normal gestation period in cattle also averages 278-284 days. Like the human, during pregnancy gonadotrophin levels remain low but serum progesterone concentration continues to build up and stays high until the last month before parturition. After the first trimester of pregnancy, oestrogen levels also begin to increase again and climb consistently until the eighth month, when they level off for about two weeks and then increase rapidly until parturition (Henricks *et al.* 1970). The most noticeable similarity for which bovine OSE can be modelled for human has been the expression profiles of localized hepatocyte growth factor (HGF) (Parrott & Skinner 2000)(Parrott & Skinner 2000), and kit legend stem cell growth factor (KL) and its receptor *c-kit* (Parrott *et al.* 2000) in OSE layer. Moreover, the extent of expression of these proteins in cultured human and bovine OSE cells were also alike. One can expect that the underlying signal transduction mechanism leading to HGF/KL-associated up-regulation of tumour-associated genes would also be similar.

This study was designed to test the hypothesis that pregnancy induces apoptosis within the OSE layer and inclusion cysts, a process mediated through over-expression of p53, which is under the control of high progesterone and oestrogen level. This was achieved by 1) measuring apoptotic cell death in inclusion cysts and OSE, 2) determining how progesterone and oestradiol affect cell death *in vitro* and 3) localization of p53 and its expression profile under progesterone influence.

4.2 Materials and methods

4.2.1 Animals

Ovaries for histological analysis and for isolation of OSE cells were obtained from pregnant and non pregnant heifers from a local abattoir. Ovaries were sorted and collected from the pregnant heifers at different stages of gestation (55-80 d, n = 8) and (90-140 d, n = 8), stage of pregnancy was determined using foetal crown-rump length. For the control groups, ovaries were collected from cycling heifers (n = 8). Gestation period in heifers lasts from 278-284 days.

4.2.2 In vivo detection of apoptosis

In situ terminal transferase-mediated dUTP nick end labelling (TUNEL) method was used in order to examine the effect of pregnancy on the apoptotic activity in the epithelial cells of the ovarian surface and inclusion cysts. Bovine ovaries were fixed in 4% paraformaldehyde and processed for histology by standard procedures (Chapter 2). According to the instructions of the commercial kit (FragEL™, DNA Fragmentation Detection Kit, Colorimetric - TdT Enzyme- calbiochem, EMD Chemicals Inc), histological sections at 5 μm were pre-treated with proteinase K (2 mg/ml proteinase K in 10 mM Tris-HCl buffer, pH8) at 37ºC for 20 min. Endogenous peroxidase activity was inactivated by incubating the slides with 30% H_2O_2 for 5 min at room temperature. The slides were then covered with an equilibration buffer (1 M Sodium Cacodylate, 0.15 M Tris, 1.5 mg/ml BSA, 3.75 mM $CoCl_2$, pH 6.6) for 30 min before incubation with TdT enzyme (Terminal Deoxynucleotidyl Transferase) for 90 min at 37ºC (labelling reaction). The reaction was stopped with the 1X stop buffer (0.5 M EDTA, pH 8) for 10 min. After washing with TBS, sections were incubated with blocking buffer (4% BSA in TBS) for 10 min. In a humidified chamber, slides were incubated with peroxidase streptavidin conjugate for 30 min. The reaction was detected with DAB in TBS containing freshly prepared 1% H_2O_2 for 10 min. Background counterstaining was done by using haematoxylin. Apoptotic nuclei stained dark brown. Negative controls were the sections incubated in the absence of TdT-enzyme. Every time the experiment was run, a

batch of sections from all the groups plus negative and positive control were included at the same time.

4.2.3 Immunolocalization of p53 and cytokeratin

In order to investigate whether the pattern of p53 distribution is related to ovulation events, sections from ovaries of non pregnant heifers (n = 8) were processed for IHC according to the procedure described in chapter 2. The monoclonal antibody (mouse anti-p53 primary antibody; Novocastra) at 1:100 dilution in PBS was used. Avidin-biotin peroxidase complex (ABC) method with DAB as chromogen was used. Negative control for each experiment was run by replacing the primary antibody with normal serum. A tissue from human ovarian tumour was used as a positive control for P53 expression. Slides were counterstained with haematoxylin. To verify the epithelial origin of the OSE cells and cells in the inner lining of inclusion cysts, sections were processed for IHC staining of cytokeratin (discussed in details in chapter 3).

4.2.4 Microscopy and data quantification

Staining density for apoptosis and p53 antigen was evaluated. Cells were analyzed under magnification of 400X. A total of 8 fields from each tissue section (4 sections per ovary) were randomly selected, and approximately 100 epithelial cells from each field were counted. All inclusion cysts observed within each section were analyzed. Data was expressed as a percentage of stained cells within the OSE layer and completely stained inclusion cysts.

4.2.5 OSE isolation and culture for in vitro studies

OSE cells were isolated from ovaries of non pregnant heifers (n = 14) by gently scraping off the cells from the ovarian surface using sterile plastic scraper. The cells were incubated in M199/MCDB 105 medium (1:1), containing 1 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% FBS. The presence of OSE flakes in the scrapings was confirmed using a phase-contrast microscope. The cells were grown in above culture medium poured in 4 culture flasks and were incubated at 37ºC under 5%

CO_2 atmosphere. Medium was changed every 3-4 days until cultures reached confluence stage (normally after 3 to 4 weeks). Once the cells had grown to confluence (Figure 4.1), the cells were harvested in trypsin /EDTA (0.05% trypsin and 0.02% EDTA) and counted using a heamatocytometer.

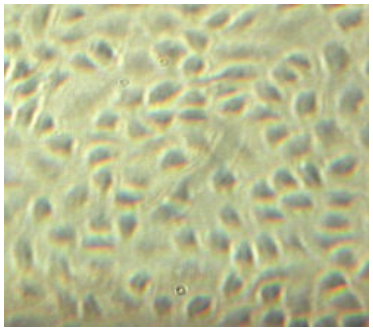


Figure 4.1 Bovine OSE cells in culture. Photomicrograph represents cultured bovine OSE cells (confluent) with a compact, cobblestone-like growth pattern, at 400X magnification.

4.2.6 Induction of apoptosis by steroid treatment

To investigate whether apoptosis in the OSE cells and inclusion cysts is controlled by steroids, an *in vitro* quantification method was used. Apoptosis was quantitatively determined using APOPercentage™ apoptosis assay kit (Biocolor Ltd., Belfast, Ireland). The assay uses a dye that is selectively incorporated into apoptotic but not necrotic cells. The incorporation of dye is considered to be due to the membrane "flip-flop" event when phosphatidyl serine is translocated from the inner membranes to the outer site.

Trypsinized OSE cells ($5x10^4$ cells/well) were seeded in 96-well tissue culture plates in 200 µl fresh culture medium supplemented with 10% serum. This was incubated for 24 h before any treatment was given. The cells were incubated at 37ºC in a humidified incubator under an atmosphere of 5% CO_2 in air for 24 h followed by 24 h incubation with serum free medium under the same conditions. After 24 h, the culture medium was removed and replaced with 100 µl/well of each treatment in the medium.

For steroid treatments, progesterone and 17β-oestradiol (Sigma-Aldrich) were administrated in two doses (30 ng/ml for the low does and 30 µg/ml for the high does). Levels of steroids in preovulatory follicular fluid reach levels of 1 µg/ml E_2 and 17 µg/ml P_4 (Lenton *et al*. 1988), it is expected that during pregnancy progesterone levels increase 100 times comparing to the normal cycle. We used the low does which represent the systemic levels (range 1 ng/ml E_2 and 30 ng/ml P_4) (Lenton *et al*. 1988) and high does that might represent the luteal progesterone during pregnancy. Progesterone and oestrogen were dissolved in absolute ethanol and supplemented to a final concentration in serum-free culture medium. For positive control, OSE cells were treated with 5 mM freshly prepared H_2O_2 (APOPercentage™ apoptosis assay kit recommendation). Control was the OSE cells incubated with medium and ethanol only. The OSE cells were then incubated for 24 h at 37ºC in 5% CO_2 incubator.

After incubation of the OSE cells in the presence of the two concentrations of progesterone and 17β-oestradiol or equivalent alcohol, the medium was discarded 30 min before the completion of the incubation. Then the cells were incubated with 200 µl of serum-free medium containing 10 µl of APOPercentage dye and these were incubated at 37 °C in humidified air supplemented with 5% CO_2 for another 1 h. After washing twice with PBS, cell surface-bound dye was extracted by lysis solution. The absorbance was measured at 590 nm wavelength using a microplate reader. All treatments were given in four replicates.

4.2.7 P53 expression at different areas of the OSE

An *in vitro* study was required in order to investigate the role of ovarian compartments in the regulation of p53 expression. OSE cells were isolated from ovaries of non pregnant heifers (n = 14) from three different locations, 1) over large antral follicles (size range 10-15 mm; n = 15), over mature corpora lutea (17 mm; n = 7) and over stroma in positions away from ovulatory activity. Sheets of OSE layer were removed by gentle scraping of the ovaries surface using sterilized plastic scraper. OSE cells were quickly removed from the scraper by placing the scraper into fresh media followed with centrifugation at 300 rpm.

4.2.8 Reverse transcription-PCR (RT-PCR) for determining p53 gene expression

The Tri Reagent method was applied to extract total RNA from suspended OSE cells. Following the manufacturer instructions, RNA samples were purified by phenol-chloroform extraction and precipitated with absolute ethanol. The amount of RNA was determined spectrophotometrically at 260/280 nm. The PCR was carried out in 50 µl reaction mixture prepared in ice bath. The reaction mixture contained 25 µl AccessQuick Master Mix, 0.5 µM upstream primer, 0.5 µM downstream primers, 1 µl AMV Reverse Transcriptase and 1 µg RNA template. The customized PCR primers used for amplification were synthesized by (Sigma-Genosys) on the basis of the available bovine sequences (Table 4.1). The PCR conditions were as follows: 45ºC for 45 min as a starting point for first strand cDNA synthesis, followed by 40 cycles at 95ºC for 2 min, 52.8ºC for 1 min, and 72ºC for 1 min, respectively, and then final extension step of 5 min at 72ºC. After amplification, 10 µl of the PCR products were separated by electrophoresis on 1.5% agarose gels containing 0.002% ethidium bromide, and visualized under a transilluminator and then photographed. The equal loading of the RNA samples was ensured by RT-PCR analysis of a housekeeping gene, *GAPDH*. Negative controls (reactions without template) were run in parallel to verify the absence of DNA contamination in RNA preparations.

Table 4.1

PCR primers for p53 and GAPDH genes, annealing temperature (Ta), number of cycles and cDNA size used for gene expression analysis.

Gene	Forward primer	Reverse primer	Ta	Cycles	cDNA size
p53	5'-CGTGTTTGTGCCTGTC	5'-GTTTACGCCCACGGAT	52ºC	40	233bp
GAPDH	5'-GTTTGTGATGGGCGTGAACC	5'-TTGGCAGCACCAGTAGAAGC	54ºC	30	255bp

4.2.9 Role of steroids in p53 regulation

To test if p53 expression in the OSE cells is regulated by steroids, an ICC assay was performed. OSE cells were obtained from culture flasks (Section 4.2.5); cells were transferred onto chamber slides ($5x10^4$ cells/well) and incubated in M199/MCDB 105 medium for 24 h at 37ºC under atmosphere of 5% CO_2 in air. This was followed by further 24 h incubation in serum free medium. Cells were treated with either progesterone (30 ng/ml and 30 µg/ml) or 17β-oestradiol (30 ng/ml and 30 µg/ml) for 24 h. For control, cells were incubated in serum free culture medium for the same period and under same conditions. After incubation, the cells were fixed with ice-cold methanol for 10 min and washed with PBS. The cells were then permeabilized by rinsing with Triton-X 100 solution in PBS, incubated for 30 min with 10% normal goat serum and for 1 h with anti-p53 antibody. After primary antibody treatment, cells were washed twice with PBS, and incubated for 30 min with secondary goat anti-mouse antibody. Slides were again washed in PBS prior to 30 min incubation with the preformed Vectastain ABC reagent. After post incubation washings with PBS, the slides were rinsed with few drops of DAB stain, and left to develop for 3-6 min. A positive reaction produces a brown endpoint, which was stopped by rinsing the slides with water. Sections were counterstained with haematoxylin, for 10-15 sec and dehydrated through graded alcohols (70% to absolute alcohol). Sections were cleared by xylene then mounted with DPX. Treatments were replicated in triplicates.

4.2.10 Statistical Analysis

The Kolmogorov-Smirnov test was used in order to test whether the data was normally distributed or not. According to the normality test that was used, the data set for *in vivo* apoptosis detection in OSE cells and inclusion cysts was not normally distributed therefore the Kruskal-Wallis test was selected as a powerful test for comparing more than two groups. Values of *in vitro* studies (induction of apoptosis and p53 expression) were presented as means of three independent assays, data was normally distributed and therefore ANOVA was selected as a robust test for analysing this type of data. After a significant ANOVA, a Tukey post-hoc test was performed in order to locate

the significant differences between the groups. Chi-square test was used to compare the total percentages of p53 expression between three different areas. Differences were considered to be significant at $P \leq 0.05$. Analysis was done using Minitab version 15.

4.3 Results

4.3.1 Cytokeratin expression in OSE layer and inclusion cysts

The epithelial cell origin of the cells lining the inclusion cysts was confirmed by their positive anti-cytokeratin immunostaining, characteristic of most types of epithelial cells (Figure 4.2). The area of the OSE around the ovary in cycling animals was completely stained with cytokeratin. Additionally, all the inclusion cysts which were distinctly detected under microscope were also expressing the epithelial marker cytokeratin. Most cysts were typically lined with a single layer of epithelial cells, flat to squamous in shape.

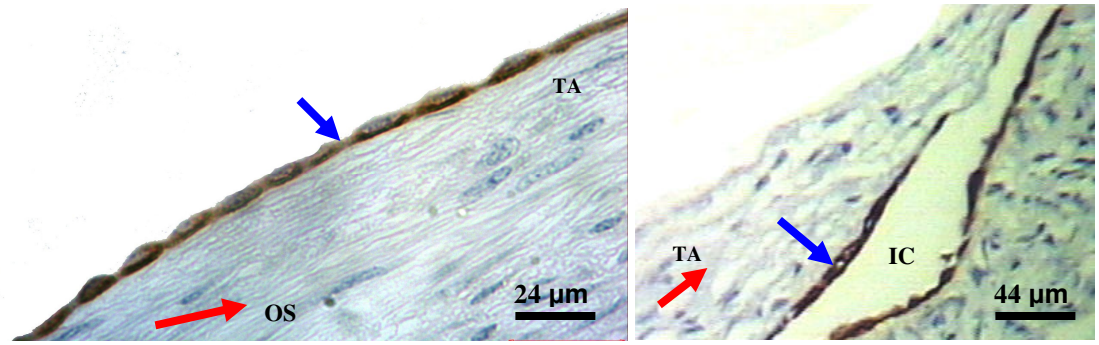


Figure 4.2 Photomicrographs representing cytokeratin expression in (A) OSE cells and (B) inclusion cyst of bovine ovaries (blue arrows). Brown staining indicates the cytokerstin expression (cytoplasmic localization). Red arrows show the negative staining in ovarian stroma (OS) and tunica albuginea (TA) layers. A at 40X and B at 200X magnifications.

4.3.2 Immunohistolocal staining of apoptotic cells in OSE and inclusion cysts in pregnant and cycling heifers

After having confirmed the epithelial nature of the OSE and inclusion cyst layers, the extent of apoptosis was enumerated in these layers at different reproductive stages. Apoptosis positive cells by TUNEL assays were recognized according to their staining patterns and morphological features such as chromatin condensation on the periphery of the nucleus, and grossly stained nucleus. Microscopic observations showed that very few apoptotic epithelial cells were discernable within the total OSE cells examined in all groups of the animals; both non-pregnant and pregnant. Tissues with relatively high apoptotic index of 6% were found in OSE cells during 55-80 d pregnancy, whereas tissues collected from 90-140 d pregnant group of animals showed significant reduction in the number of apoptotic OSE cells in proportion to total cells (2%) ($P \leq 0.05$). The least apoptotic index was found in the OSE cells of non-pregnant group (0.8%) ($P \leq 0.05$) (Figure 4.3).

When inclusion cysts were considered for such analysis, a similar trend of apoptotic index was observed within epithelial cells of different group of animals as was found in case of the OSE cells. Data analyses showed that 37% of the inclusion cysts in the ovarian tissues from 55-80 d pregnancy animals were completely apoptotic. In fact under the microscope all the lining cells seemed to be apoptotic (Figure 4.4). Conversely, the apoptosis analysis through the ovarian tissues from 90-140 d pregnant animals indicated that the number of completely apoptotic inclusion cysts was reduced to 10% ($P \leq 0.05$). Interestingly, the microscopic observations showed that the vast majority of inclusion cysts in the ovarian tissues of non-pregnant animals were completely healthy with less than 2.5% of them lined with flattened apoptotic cells.

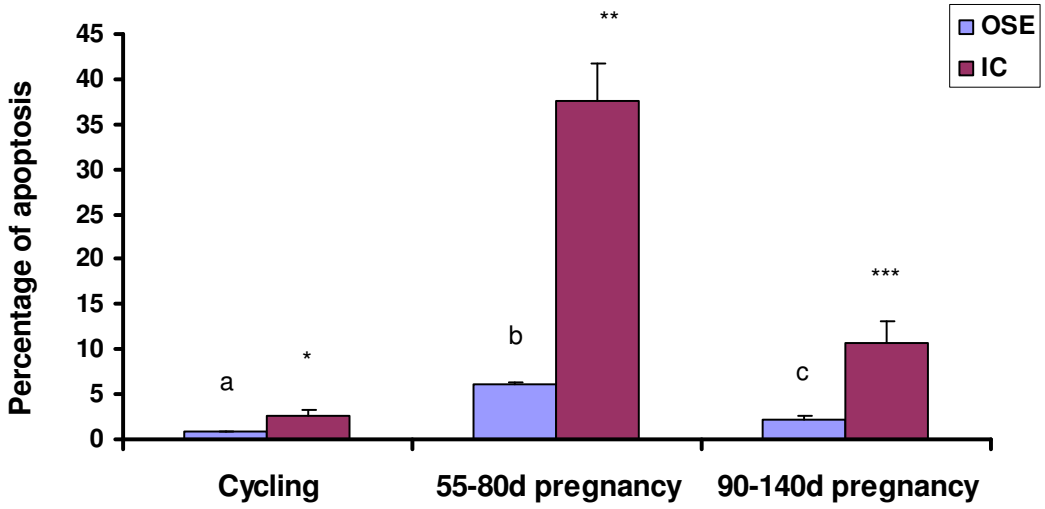


Figure 4.3 Histogram representing *in situ* localization of apoptotic cells within the OSE cells and inclusion cysts at cycling (n = 8), 55-80 d pregnancy (n = 8) and 90-140 d pregnancy (n = 8) groups. Different letters above the bars indicate statistically differences between groups among OSE and asterisks represent statistical difference between groups among inclusion cysts ($P \leq 0.05$).

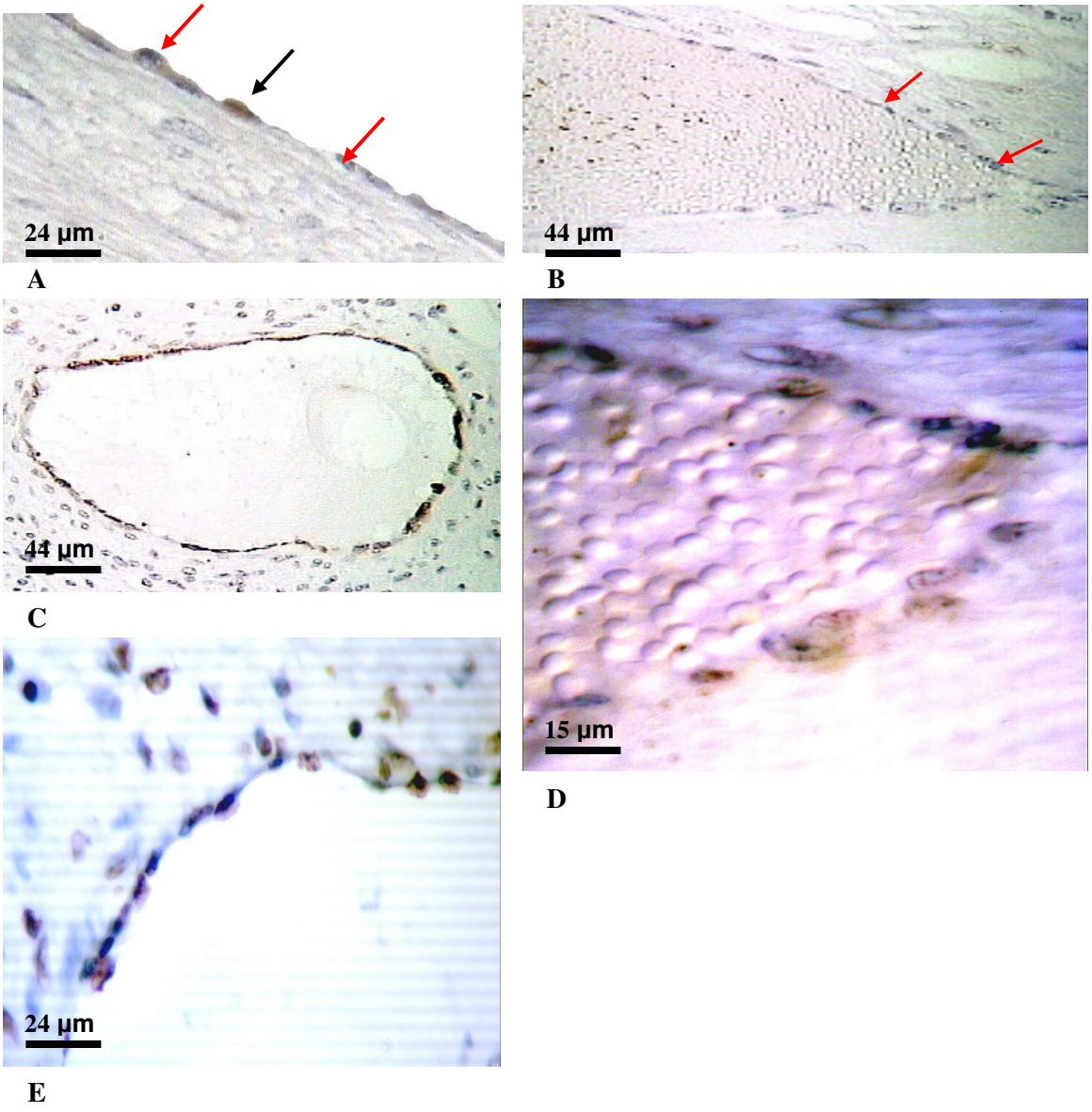


Figure 4.4 Photomicrographs representing apoptotic cells in bovine OSE layer and inclusion cysts. (A and B) show apoptotic cells in OSE and inclusion cyst of cycling group, (black arrows show the apoptotic cells with brown stained nuclei and red arrows show the negatively stained cells). (C) Shows the TUNEL labelling in the inclusion cyst of mid pregnant group (completely stained). D is a high-power view of B and E is a medium-power view of C.

4.3.3 Induction of apoptosis by progesterone in cultured OSE cells

APOPercentage™ assay, a colorimetric apoptosis detection system was used to detect apoptotic cell death in the cultured OSE cells upon treatment of two concentrations of progesterone and 17β-oestradiol. After 24 h of 30 µg/ml progesterone treatment, as shown in (Figure 4.5), the level of apoptotic cells in population of total OSE cells reached 2-fold relative to the control (untreated cells). Under the same conditions, level of cell apoptosis was approximately 1.1-fold in OSE cells treated with low dose of progesterone (30 ng/ml). Treatment of OSE cells with low dose of 17β-oestradiol (30 ng/ml) did not induce apoptosis when compared with controls OSE cells incubated with equivalent ethanol. On the other hand, treatment with high dose of oestrogen (30 µg/ml) resulted in 1.6-fold increase in apoptosis, which was similar to the result of low dose of progesterone.

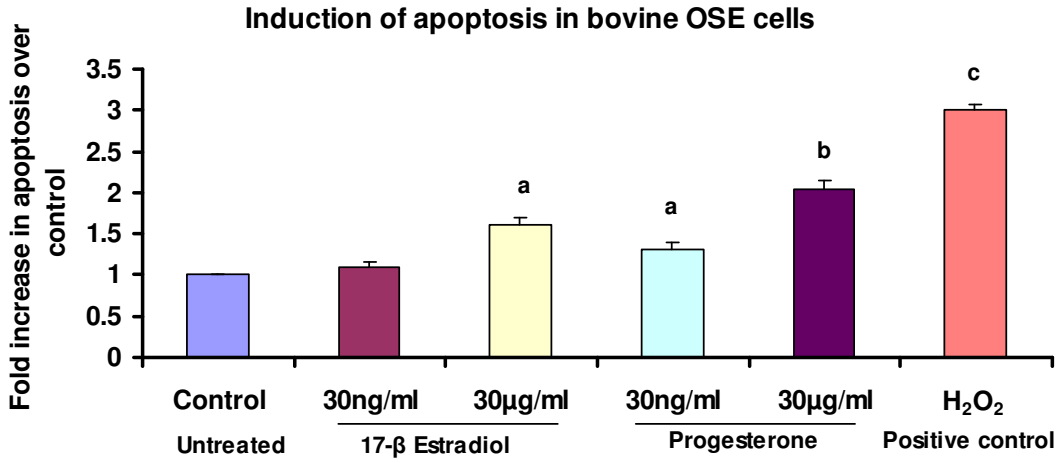


Figure 4.5 Histogram representing apoptosis in bovine cultured OSE cells after incubation with different treatments for 48 hours. OSE cells were incubated without treatment (control) or in the present of steroids (17-β oestradiol or progesterone) at different doses (30 ng/ml and 30 µg/ml) or incubated with 5 mM H_2O_2 (positive control for apoptosis). Values for each treatment are expressed relative to the values of the control, which was taken as unity (1). Different letters over bars indicate statistical differences between each treated group and control ($P \leq 0.05$). Data expressed as a mean ± SEM; (three independent assays).

4.3.4 Effect of steroids on p53 expression in cultured OSE cells

Since the results of cellular apoptosis revealed a direct influence of progesterone and to some extent oestrogen, we wanted to determine whether this process was associated with increased expression of p53 in the same cells. To study the effect of progesterone and oestrogen on p53 expression, cultured OSE cells were treated with oestrogen and progesterone at the doses 30 ng/ml and 30 µg/ml. Results from ICC revealed that low dose (30 ng/ml) of both the steroid treatments did not induce p53 expression. However, a high dose of progesterone (30 µg/ml) increased p53 expression significantly over controls ($P \leq 0.01$). Oestrogen treatment at high dose 30 µg/ml also increased the level of p53 expression significantly ($P \leq 0.05$) but to a lesser degree than the high dose of progesterone (Figure 4.6).

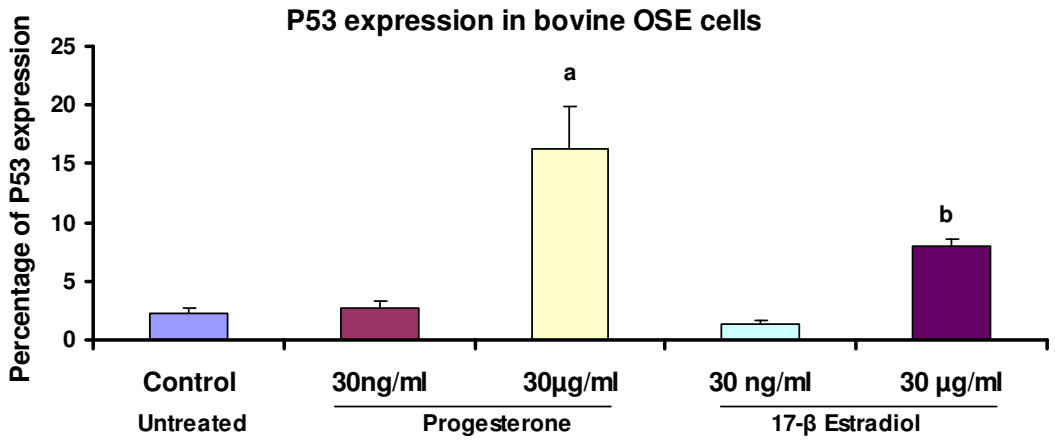


Figure 4.6 Histogram representing the percentage of p53 immunostaining after steroid treatments in cultured bovine OSE cells. Cultured OSE cells were incubated without treatment (control) or in the present of steroids (progesterone and 17-β oestradiol) at different doses (30 ng/ml and 30 µg/ml) for 24 h. Control cells were incubated with serum free medium under the same conditions. Different letters over bars indicate statistical differences between each treated group and control ($P \leq 0.05$). Data expressed as a mean ± SEM; (three independent assays).

4.3.5 P53 Immunohistolocalization in cycling animals

Since both apoptosis induction and p53 expression were regulated by direct hormone treatments, we aimed to detect p53 expression in different regions of OSE layer in cycling animal. Microscopic observations showed that p53 expression within the nuclei of the OSE cells was very low in most of the areas around the ovary. Analyzing the labelling index in each area of the OSE layer; over the large antral follicles, over the CL and over the stroma away from ovulation events showed, that more immunoreactive cells were detected over CL than in the other areas. The labelling index of p53 antigen was 4% over the CL, which was significantly higher than the number of labelled cells, which detected over the large antral follicles (1%) ($P \leq 0.05$). No positive stained cell for p53 was detected in the OSE cells over the stroma (Figure 4.7 and 4.8).

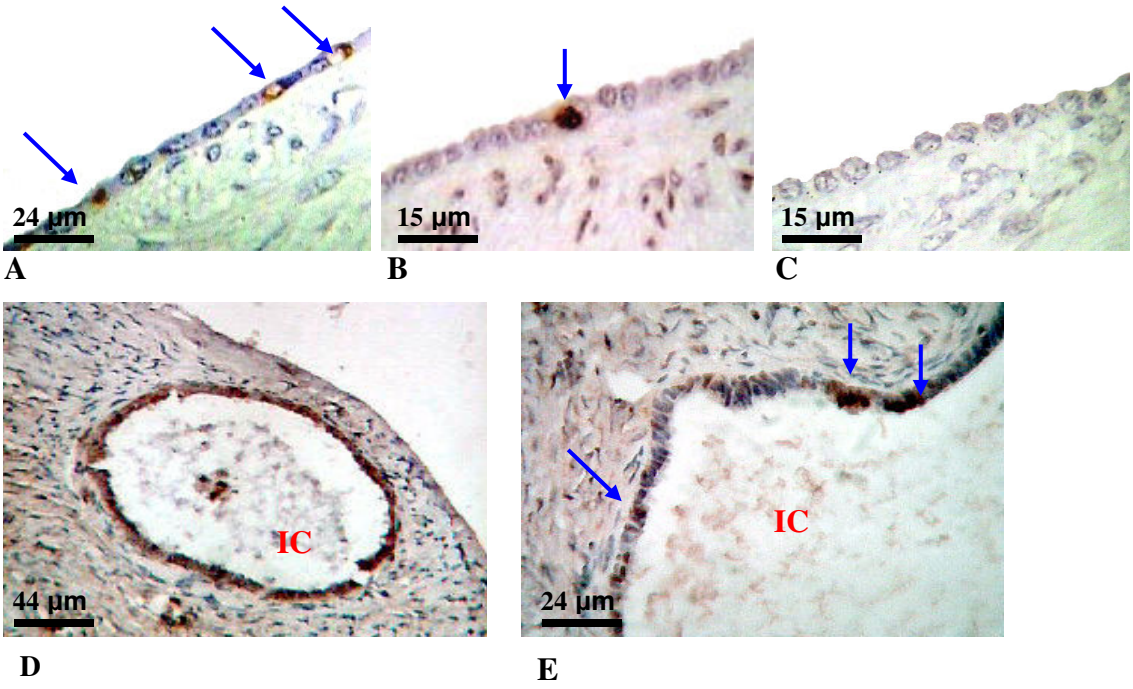


Figure 4.7 Photomicrographs showing p53 immunostaining within the nuclei of OSE cells over different areas of the ovary. P53 expression in the OSE cells over the (A) CL, (B) large antral follicle and (C) ovarian stroma. Blue arrows show the positively stained cells (Brown nuclei). Panel D and E show positive control for p53 immunostaining in cells lining the inclusion cysts (IC) of human ovarian tissues, A and E at 400X, B and C at 600X and D at 200X magnifications.

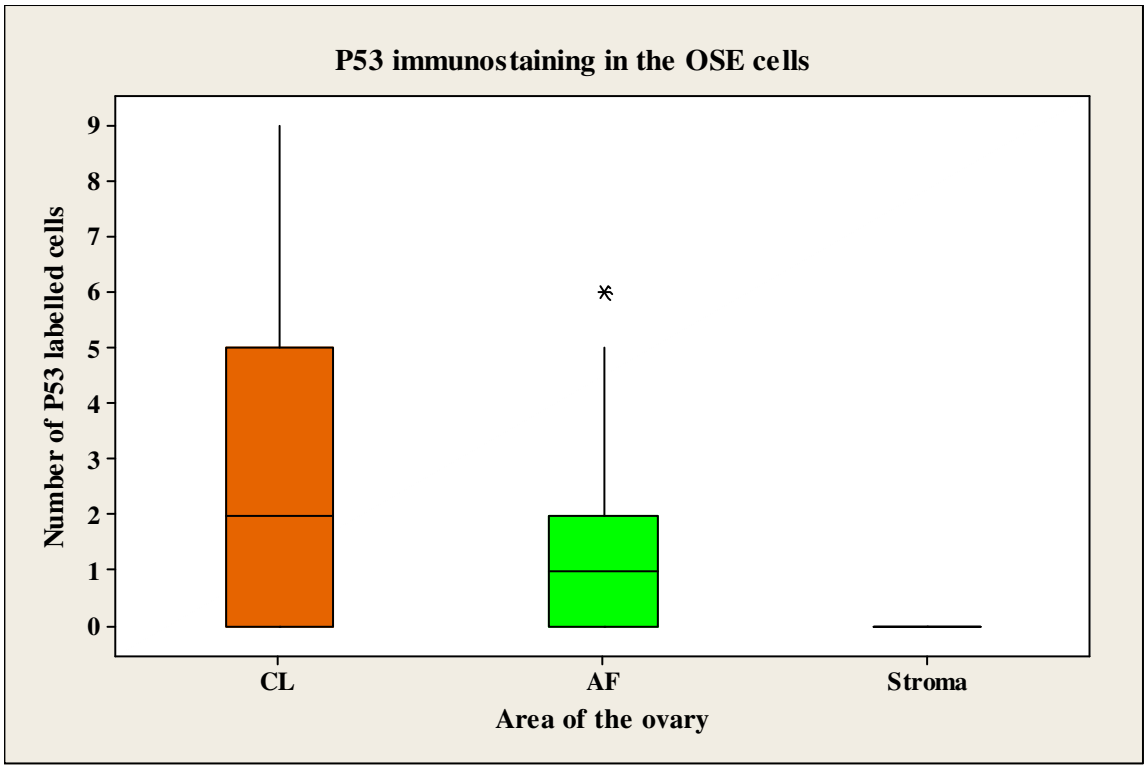


Figure 4.8 Box plots representing P53 immunohistochemistry in the OSE cells of cycling heifers (n = 8) through different regions of the ovary (overlying corpora lutea (CL), antral follicles (AF) and stroma). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Data are the number of OSE labelled cells over each region for each section (3 sections/animal). Total number of counted OSE cells over different regions of the surface is presented in Table 4.2.

Table 4.2

P53 labelling in OSE cells of cycling heifers

Animal	CL		AF	
	No. labelled cells	**No. unlabelled cells**	**No. labelled cells**	**No. unlabelled cells**
1	5	795	0	800
1	7	793	0	800
1	2	798	0	800
2	0	0	2	792
2	2	798	0	800
2	0	800	1	799
3	3	797	1	799
3	3	797	2	798
3	5	795	1	799
4	8	792	0	800
4	4	796	0	800
4	6	794	0	800
5	6	794	0	800
5	9	791	1	799
5	2	798	1	799
6	0	800	2	798
6	0	800	0	800
6	0	800	0	800
7	3	797	0	800
7	2	798	1	799
7	4	796	1	799
8	5	795	0	800
8	1	799	1	799
8	0	800	1	799

4.3.6 P53 gene expression in different region of the OSE layer in cycling animals

To understand whether differential p53 expression at different OSE regions were due to higher turnover of the pre-formed p53 protein or *de novo* synthesis, the *p53* gene expression was monitored at mRNA level by RT-PCR analysis. Results indicated that the level of *p53* gene expression of the OSE cells collected over the CL was higher than the equivalent cells from other areas. The *p53* gene expression of the OSE cells collected from over the large follicles was lower than corresponding values found over the CL. There was no apparent *p53* gene expression observed in the OSE cells collected from over the stroma.

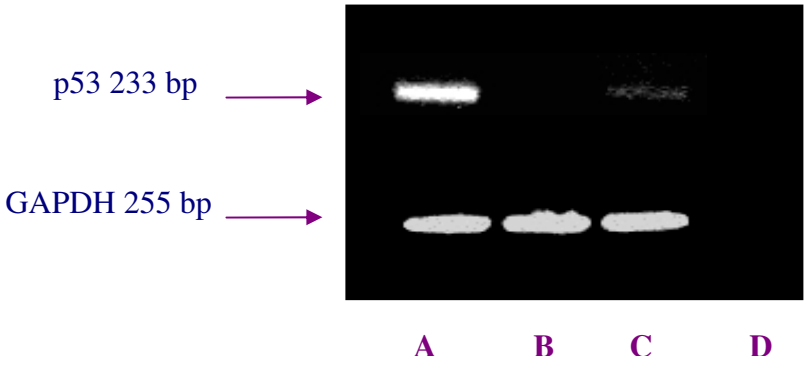


Figure 4.9 PCR gel showing *p53* gene expression of freshly isolated bovine OSE cells. OSE cells were collected from different regions of the ovaries (n = 14) in relation to the underlying compartment. Lane A. OSE cells overlying corpora lutea (n = 8), lane B. ovarian stroma, lane C. antral follicles (n = 15) and lane D. negative control. Total RNA was reverse transcribed and amplified by PCR using p53 specific primers (upper panel). Detection of GAPDH mRNA transcripts served as a positive control for amplification (lower panel).

4.4 Discussion

Elevated progesterone concentration during pregnancy and use of progesterone-like contraceptives are known to reduce ovarian cancers (Risch 1998). This study was undertaken to investigate whether or not there is a relationship between progesterone (also oestrogen)-mediated OSE apoptosis and expression of p53. The results of the current study concluded that progesterone during cycling and pregnancy plays a role in suppressing ovarian cancer by ceasing cell cycle and diverting damaged and mutagenized OSE cells for apoptosis, and the process may be mediated through elevated p53 synthesis. However, it is also possible that progesterone and p53-induced apoptosis may be entirely different cancer suppressive actions but coincidently happening together.

The immunohistochemical analysis of the tissues revealed that cytokeratin staining was present only in the surface epithelia. Anti-cytokeratin antibody used in this test recognizes only the epithelial cells' cytoplasmic microfilaments and not the mesenchyma, which might arise due to epithelial-mesenchymal conversion after ovulation. Due to the fact that the inclusion cysts were also stained, we rule out any possibility of invasion by fibroblast-like mesenchymal cells in the cysts. The inclusion cyst epithelium is the preferred site for metaplasia transformation and tumourigenesis, and epithelial-mesenchymal transformation is a mechanism to prevent this manifestation. Since the objective was to demonstrate that apoptosis could be a mechanism to avert malignancy in these tissues, it was necessary to verify that the cells were of epithelial origin and not mesenchymal. Using standard TUNEL assay which recognizes apoptotic nuclei, very high percentage of epithelial cells from inclusion cysts during the pregnancy period (55-80 days) acquired the staining. Although the percentage of apoptosis-positive cells was much lower in the OSE layer than in the inner lining of inclusion cyst, the highest frequency in this tissue was also seen during 55-80 days pregnancy period. There were negligible cells with nuclear stain in the cycling animals, suggesting that practically no apoptosis took place in the oestrus period. The frequency of apoptotic cells started to decrease as the animals approached 90-140 days pregnancy. This observation was rather unexpected because the serum progesterone level reaches its

maximum during late pregnancy period (Smith *et al.* 1973). This indicates that serum hormone level may not reflect the actual concentration of progesterone in the vicinity of the ovarian surface. Until mid pregnancy, the CL produces progesterone, and when it starts to regress, the placenta takes over. Because of the proximity, the effective hormonal concentration around the OSE is expected to be high under the influence of the CL rather than placenta, even though both of them might contribute some progesterone level in the blood stream. Once the CL starts to regress, that is during mid-pregnancy, the effective concentration starts to decline.

To ascertain a direct effect of steroidal hormone on apoptotic response on OSE, the cells were cultured in M199/MCDB 105 medium with standard additives. For bovine cells there seems to be some flexibility in choice of medium, because for culture purposes Dulbecco's modified Eagle's medium has also been used by some workers (Parrott & Skinner 2000). One concern with OSE cell culture is that through growth passages, epithelial cobblestone compact cells tend to convert to spindle shaped mesenchymal phenotype (fibroblast-like shape) (Auersperg *et al.* 2001). The cultivated bovine OSE cells maintained usual cobblestone morphology of original source cells of OSE layer, and probably the genetic makeup also remained unchanged. There were two noteworthy findings with the cultured cells. Oestrogen at high and progesterone at both high and low concentrations significantly increased the *in vitro* apoptotic activity. In fact, high progesterone stimulated apoptosis to nearly the same extent as positive control (H_2O_2-treatment). Besides, both hormones also induced expression of cell-cycle arrester and apoptosis-inducer protein, p53. We propose that progesterone, and to some extent oestrogen, through their receptors may up-regulate a cascade of downstream pathways leading to cell-cycle arrest and programmed cell death, and further that p53 protein seems to be responsible as a mediator molecule. Progesterone and oestrogen receptors have been localized within the bovine OSE cells (D'Haeseleer *et al.* 2007; D'Haeseleer *et al.* 2006; van den *et al.* 2002).

The results of *in vitro* hormonal effects paved the way to test the actual *in vivo* influence. For this, p53 expression at mRNA and protein level was examined in those regions of OSE layer that are directly in contact with stroma, large antrum follicle or CL. The CL is the source of progesterone in cycling animals, and one can expect that OSE in

its proximity would be under the progesterone influence. Antral follicle and CL are also the source of oestrogen and they might also affect the neighbouring OSE layer. In this situation p53 expression should be high in only those regions of OSE which are in contact with the CL or antrum. Accordingly, we found a very high extent of p53 immunoreactivity and PCR product of *p53* gene mRNA in OSE layer overlying CL. The control, GAPDH expression was nearly identical in all the cells. There was some marginal expression of p53 protein in OSE overlying antral follicles, which is most likely due to oestrogen release. Although not tested, we hypothesize that in the cow, induced apoptosis during mid pregnancy phase is due to elevated progesterone, and is perhaps mediated through p53. The relationship between progesterone induced anti-cancerous effects in OSE layer and p53-mediated apoptosis and thereby evading tumourigenesis may be purely coincidental. This is because the signals inducing p53 expression are mitogenic and/or DNA damaging activities (Corney *et al.* 2007) rather than steroids.

Wilcox *et al.* (2007) for the first time elucidated a direct contact effect of progesterone on anti-cancerous properties of cultured human OSE cells. The approach was microarray-based transcriptional profiling of over 22,000 transcripts (cDNA's generated from total RNA's) of progesterone treated and un-treated control cells. The most significant up-regulation in treated cells was of the enzymes and transporter proteins of cholesterol biosynthetic pathway, besides the progesterone receptors, PR-A and PR-B. Among the cholesterol biosynthesis genes, *TMEM97* gene encoding a trans-membrane protein of unknown function was found to be most expressed. It was concluded that progesterone through its receptors mediate an up-regulation of cholesterol and lipid biosynthesis in OSE cells. This in turn decreases the cell membrane fluidity, and such cells are less vulnerable to inflammatory and physical damage due to ovulation.

Another microarray analysis was carried out to elucidate *p53* gene targets in cultured mouse OSE cells (Corney *et al.* 2007). For this, microRNA (miRNA) expression of OSE cells in *p53* knock-out mutant and wild type mice was compared. It was found that two genes for miRNA's, termed *miR34-b* and *miR34-c* were over-expressed in wild type cells. Upon induction of *p53* gene these two miRNA's also over-

expressed. These miRNA's were shown to "silence" several cancer-related genes like *Delta-like 1*, *Notch-1*, *Met*, *Myc*, *Cdk-6*, *Cyclin D1* etc. Mi-RNA-mediated repression of these downstream target genes was considered to be a mechanism whereby *p53* suppresses the tumour progression. Comparative genome hybridization and microarray profiling of normal and neoplastic OSE cells was carried out to elucidate signature genes for tumour suppression and many of them were found to be involved with the p53 pathway (Landen *et al.* 2008). Mutation of *p53* gene and some other ovarian and breast tumour suppressor genes like *BRCA1* and *BRCA2* are linked to "high-grade pathway" of malignancy due to unchecked proliferation, inhibition of apoptosis, angiogenesis, stromal invasion, separation and survival away from the primary tumour, and implantation and growth within new tissues.

In conclusion, progesterone and p53 seem to be associated with suppression of bovine EOC in both cycling and pregnant animals. At least two distinct mechanisms may be attributed to this phenomenon; 1) anti-inflammatory activity due to progesterone-mediated increase in cholesterol and lipid biosynthesis, and 2) p53-mediated cell cycle-arrest and apoptosis. Whether these attributes are connected to each other or whether they are two distinct events independently contributing to tumour suppression, needs to be further investigated in future.

CHAPTER FIVE

Changes in the Proliferation Activity of the Ovarian Surface Epithelium during the Ovulatory Cycle, Pregnancy and after GnRH-Antagonist Treatment in the Primate

5.1 Introduction

Gonadotrophin releasing hormones (GnRHs) are decapeptides produced from specialized neurosecretary cells in the hypothalamus, targeting the pituitary-gonadal axis. Over 20 different forms of GnRH in vertebrate species have highly conserved structure (Millar 2005). In mammals, three forms are known, GnRH-I (mammalian GnRH), GnRH-II (chicken GnRH-II) and the salmon GnRH (RnRH-III) (Metallinou *et al.* 2007). GnRH-I is a typical pituitary controlling hormone, with affinity to the pituitary seven transmembrane domain G protein ($G_{q/11}$)-coupled receptors (Stanislaus *et al.* 1997). After binding, GnRH-I modulate membrane phospholipase C (inositol phosphate) and calcium channel, and consequently stimulate LH synthesis and secretion. A direct control over FSH secretion has not been unequivocally proven. Plasma gonadotrophin levels regulate the overall reproductive process, especially the gonadal steroidogenic and gametogenic functions (Millar 2005). The GnRH-I and II receptors are also found in almost all types of normal and cancerous epithelial cells, including those present in gonads (Millar *et al.* 2001). The extra-pituitary function of these receptors in these tissues is not fully understood, but evidence suggests that the GnRH-I and II signal transduction pathways in epithelial tissues are different from pituitary cells (Emons *et al.* 2003). Interestingly, GnRH-II receptors in these epithelia have conserved regions of GnRH-I and binding of the two GnRHs to their receptors can be mutually exclusive (Mamputha *et al.* 2007). Studies suggest that GnRH-II has a role in suppression of cell proliferation and apoptosis by triggering a G-protein (G_i) mediated signal pathway in the target cells (Wu *et al.* 2009).

During the last four decades, several thousand peptide and non-peptide GnRH agonists and antagonists have been synthesized and tested for clinical efficacy (Fister *et al.* 2007; Agorastos *et al.* 2004). These compounds, which are generally GnRH analogs, competitively bind to pituitary GnRH receptors and by two distinct mechanisms down-regulate gonadotrophin synthesis and secretion. Within 7-14 days of a GnRH agonist administration, a “surge” of gonadotrophins and steroidal hormones, known as “flare”, is followed by a rapid fall in their serum concentrations. In contrast, the antagonists lower the hormone concentrations from the beginning. Both groups of synthetic analogs also

down-regulate expression of GnRH receptors and initiate internalization of the cell surface receptors to the nuclear membrane (Herbst 2003). This further hinders the binding of GnRH to pituitary cells. Clinically, the agonists/antagonists are used for the treatments of prostatic, breast and endometriotic cancers, uterine fibroids, precocious puberty, premenstrual and polycystic syndromes, and infertility (Emons *et al.* 2003; Millar 2005; Hayden 2008). Among several GnRH antagonists developed, Antarelix was formulated in the 1990's and extensively used as a polypeptide GnRH-I antagonist (Deghenghi *et al.* 1993). The advantage of this compound is its high water solubility, modest allergy and histamine stimulation and high potency.

Because they have similar patterns of serum β-oestradiol, progesterone, testosterone, LH/CG during the ovulatory cycle and pregnancy to that of the human being, nonhuman primates are used as experimental models to study hormone-related reproductive problems like anovulatory infertility (Steinetz *et al.* 1995; Abbott *et al.* 2004). Among primates, the reproductive physiology of Old World (e.g. rhesus; *Macaca mulatta*) and New World (marmosets; *Callithrix jacchus*) monkeys differs. Some ubiquitous features of marmosets distinct from higher primates are: 1) multi-ovular species with ovulatory cycles of ~28 days, comprising short follicular (8-9 days) and long luteal (19 days) phases, 2) ovulation rates ranging from one to four follicles per cycle (Gilchrist *et al.* 2001), 3) pituitary gonadotropes secret chorionic gonadotrophin (CG) and not LH, 4) LH receptors bind to CG and course of action of two hormones is similar (Tannenbaum *et al.* 2007), and 5) ovarian senescence is more subtle and does not resemble the menopause in women. Like humans, the follicular phase is characterized by low level of progesterone, rising oestradiol and typical pre-ovulatory "surge" of LH (Harlow *et al.* 1984). Marmoset ovaries contain a multitude of small antral follicles and towards the end of the follicular phase; two or three antral follicles enlarge and go on to ovulate. There exists clear morphological and size distinction between large ovulating and small non-ovulating antra (Gilchrist *et al.* 2001). Ovarian epithelial proliferation is believed to be primarily regulated at the level of gonadotrophins, which stimulate follicular oestradiol production. As a mitogen, β-oestradiol and other localized factors such as insulin-like and hepatocyte-growth factors (IGF, HGF) trigger proliferative response in post-ovulatory OSE layer at the sites of rupture. Poly-ovulatory mammals

like marmosets have repeated ovulations and therefore repeated rupture and healing of the OSE layer. These features accelerate OSE cell multiplication and transformation to neoplasia at the rupture sites. Notwithstanding, gonadotrophin-associated incidences of ovarian cancers are rarely reported in marmosets, even though EOC affects only primates and very rare other mammals. It is likely that the 10-fold higher progesterone concentration in pregnant marmosets compared to other primates (Steinetz *et al.* 1995) may help suppress the OSE tumourigenesis in these animals.

Antarelix GnRH antagonist has been used in marmosets to suppress LH/CG secretion from the pituitary gland, and the follicular and luteal development was examined in such treated animals (Taylor *et al.* 2004). It was found that upon such treatment the level of serum β-oestradiol rapidly falls and as a result proliferation of granulosa cells in antral follicle and CL diminishes. Follicular vasculature remained underdeveloped and the resultant follicle's growth and differentiation was restricted and ovulation was unsuccessful (Taylor *et al.* 2004). The GnRH antagonist may also suppress the mammalian OSE proliferation and tumourigenesis in a dualistic manner. First, it may do so by inhibiting LH/CG secretion so that follicular development is inhibited and ovulation is prevented. Hence the number of re-epithelization events is reduced and OSE stays non- proliferative. Second, the antagonists may bind directly to OSE cell's GnRH receptors and impart anti-proliferative response through some unknown downstream signaling pathway. Another action could be that reduced LH-controlled steroidogenesis reduces the production of localized pro-proliferative mitogens (β-oestrogen) in granulosa cells and other inflammatory agents which cause genetic damage to the epithelial cells.

According to the gonadotrophin theory of ovarian cancer, a high level of gonadotrophins at menopause has a role in the development of epithelial ovarian cancer. In this study, we test the hypothesis that GnRH antagonist administration would suppress endogenous gonadotrophin, resulting in inhibition of OSE proliferation in marmoset. We also hypothesize that changes in the ovarian environment during the ovarian cycle (follicular and luteal phase) and at early pregnancy might have an influence on the activity and morphology of the OSE cells. To our knowledge this is the first report of a GnRH antagonist treatment on OSE proliferation in a primate.

5.2 Materials and methods

5.2.1 Animals and tissue samples

Adult female common marmoset monkeys (*Callithrix jacchus*) (age 2-3 yr) with body weight ~350 g, and having a regular 28 d ovulatory cycles or gestation periods between 141 d and 145 d, were housed in cages under standard conditions. The ovaries used for this study had been collected for analysis on day 0 (n = 4), day 5 (n = 5) and day 10 (n = 6) of the follicular phase (Taylor *et al.* 2004), and at early (n = 5), mid (n = 5) and late (n = 5) luteal phase (14-22 d) of the ovarian cycle (Wulff *et al.* 2001; Rowe *et al.* 2002). Additionally, ovaries from the pregnant animals were collected from second (n = 5), third (n = 3) and fourth (n = 6) week of pregnancy (Rowe *et al.* 2002).

5.2.2 Treatments

In order to synchronize the timing of ovulation, marmosets were treated intramuscularly with a single *i.m.* injection of 1 µg of a prostaglandin (PG) $F_{2\alpha}$ analogue (cloprostenol, Planate; Cheshire, UK), between days 12-15 of 20 d luteal phase to induce luteolysis. The day of the PG injection was designated as follicular day zero. A GnRH antagonist Antarelix (Europeptides, Argenteuil, France) was dissolved in water to reach a concentration of 10 mg/ml. To provide a slow-release depot, Antarelix was administrated at a dose of 12 mg/kg body weight by subcutaneously injecting on follicular day 0 (day of PG injection) (n = 5) or day 5 (n = 5) (Taylor *et al.* 2004). Ovaries were collected on day 10 following PG administration, corresponding to the periovulatory period in the control animals. Control marmosets were studied in parallel on day 0 (n = 5) and day 10 (n = 5) corresponding to PG administration. All animals were injected *i.v.* with 20 mg BrdU (Roche Molecular Biochemicals, Essex, UK) prepared in saline. After 1 h they were killed with an *i.v.* injection of 400 µl of Euthetal (sodium pentobarbitone; Rhone Merieux, Harlow, Essex, UK). Ovaries were removed, weighed and immediately fixed in 4% neutral buffered formalin. After 24 h, the ovaries were transferred to 70% ethanol, dehydrated and embedded in paraffin. The ovary sections were prepared and processed for immunohistochemical analysis as described (Wulff *et al.* 2001; Rowe *et al.* 2002).

5.2.3 Immunohistochemistry

The changes in the proliferative activity of the OSE throughout the follicular phase, luteal phase and early pregnancy and after GnRH antagonist treatment were studied by quantifying the number of proliferating cells within the OSE area whose nuclei were stained with BrdU. For this, sections were prepared on glass slides and stained for BrdU as described previously (Taylor *et al.* 2004; Wulff *et al.* 2001; Rowe *et al.* 2002). For BrdU immunostaining, antigen retrieval was performed by pressure cooking using a Tefal Clypso pressure cooker (Tefal, Langley, Berks, UK). Sections in 0.01 M citrate buffer, pH 6, were kept for 6 min at high pressure setting (2). Slides were then left for 20 min in hot buffer and washed in Tris-buffered saline (TBS; 0.05 M Tris-HCl and 1.5 M NaCl). To reduce non-specific binding, sections were blocked in normal rabbit serum (NRS; 1:5 diluted with TBS containing freshly added 5% bovine serum albumin) for 30 min. Primary antibody, BrdU (mouse anti-BrdU; Roche Molecular Biochemicals, Essex, UK) was diluted 1:30 in TBS. Incubation was carried out overnight at 4ºC. Slides were washed three times in TBS. Incubation with the secondary antibody rabbit anti-mouse IgG (1:60 diluted in NRS: TBS; DAKO Corp.) was carried out for 40 min at room temperature, followed by two washings in TBS. and incubation with alkaline phosphatase-anti-alkaline phosphatase complex (1:100 dilution in TBS; DAKO Crop.) for 40 min at room temperature. Slides were visualized using 500 µl nitro blue tetrazolium (NBT) solution containing 45 µl NBT substrate (Roche Molecular Biochemicals), 10 ml NBT buffer, 35 µl 5-bromo-3-chloro-3-indolyl-phosphate and 10 µl levamisole. Sections were counterstained with haematoxylin. The positive immunostaining of BrdU within the granulosa cells of growing follicles was used a s a positive control, figure 5.1 shows photomicrographs of antral and preantral follicles with high levels of BrdU labelling comparing to the OSE cells (Page 114).

5.2.4 Quantification of immunohistochemistry

BrdU incorporation of the OSE showing proliferating cells was quantified. Proliferation of the OSE was detected all around the circumference and in all sections. At least 20 sections for each group of study were examined. The labelling index was

determined by counting the number of OSE cells (labelled and unlabelled) in each section under 600X magnification, and expressed as the percentage of labelled OSE cells. The labelling index is expressed as a mean value for the number of OSE cells in each section.

5.2.5 Statistical analysis

The Kolmogorov-Smirnov test was used in order to test whether the data is normally distributed or not. Data for studies of GnRH antagonist treatment and the ovarian cycle influences on OSE cell proliferation was not normally distributed therefore; The Kruskal-Wallis test was selected in order to compare different groups.
Data for BrdU labelling distribution over different areas of the ovary was not normally distributed, therefore Chi-square test was used to compare the total percentages between groups. Differences were considered to be significant at $P \leq 0.05$. Analysis was carried out using Minitab version 15.

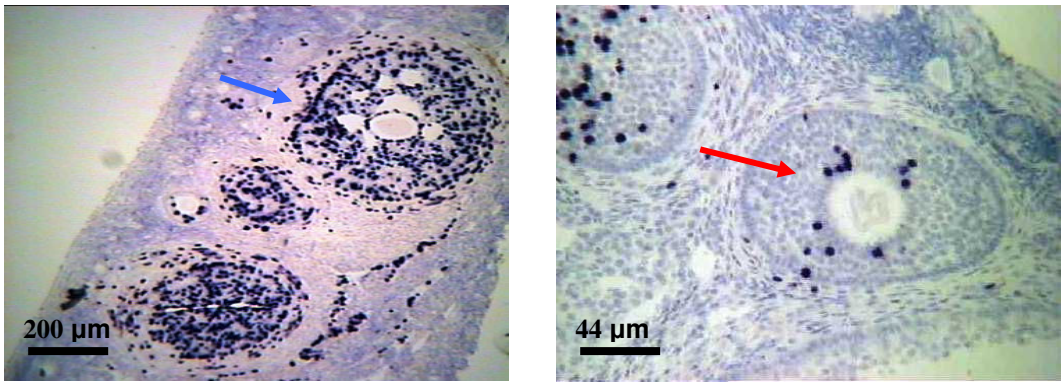
5.3 Results

5.3.1 OSE proliferation at different stages of the ovarian cycle and during early pregnancy

Results from this analysis revealed that the highest proliferation was obtained during the late follicular and early luteal phase. There was a gradual increase in the proliferation activity as the follicular phase advanced, reaching the maximum value towards the end of this phase ($P \leq 0.05$) (Figure 5.2). After ovulation, proliferation in early luteal phase ovaries was also high before declining markedly by the mid luteal phase ($P \leq 0.05$). This low level was maintained in late luteal phase ovaries.

To ascertain which underlying tissue is contributing to the OSE proliferation, the BrdU staining was examined at different regions of the OSE layer. During the follicular phase the majority of the proliferating cells were detected over the large antral follicles which were very close to the surface (60-75 μm), and a lower number of proliferating cells were found distributed randomly over the stroma. During the luteal phase, most of the proliferating cells were located over the CL and little activity was observed over the stroma (Figures 5.3 and 5.4).

During all stages of pregnancy (2, 3 and 4 weeks), the OSE cells showed diminished or extremely low BrdU labelling, and correspondingly very low labelling index as compared to the non-pregnant cycling animals ($P \leq 0.05$). The OSE proliferation was discernable mostly at week 2 of pregnancy (Figure 5.2). During early pregnancy a variation in the distribution of the proliferating cells within the OSE was also detected. The highest number of proliferating cells was found over the CL, whereas slightly lower number of evenly distributed cells was observed over stroma. At this pregnancy stage, large antral follicles were not detected close to the ovarian surface (Figures 5.3 and 5.4).



A **B**

Figure 5.1 Photomicrographs showing BrdU labelling within the granulosa cells of growing follicles in marmoset ovary. Histological sections at (A) follicular phase of the ovulatory cycle (blue arrow indicates high level of BrdU labelling in granulosa cells of antral follicle) and (B) at pregnancy (red arrow indicates low level of BrdU labelling in granulosa cells of preantral follicle), A at 40X and B at 200X magnifications.

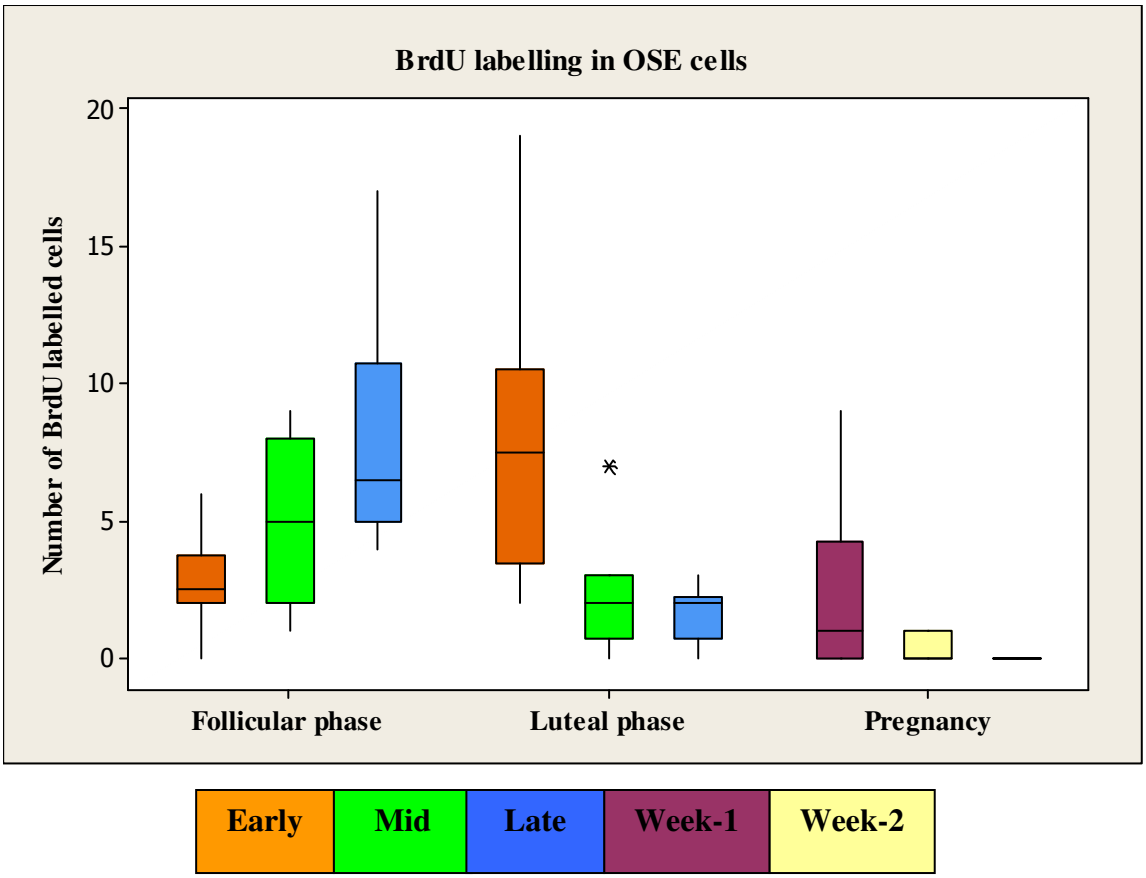


Figure 5.2 Box plots indicating BrdU incorporation into the marmoset OSE cells over the total area of the ovary at early (n = 4), mid (n = 5) and late stages (n = 6) of the follicular phase, and early (n = 5), mid (n = 5) and late stages (n = 5) of the luteal phase and at week-1 (n = 5) and week-2 pregnancy (n = 3). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Data are the number of BrdU labelled cells in OSE cells in each section (2 sections /animal). Total number of counted cells is presented in Table 5.1, 5.2 and 5.3.

Table 5.1

BrdU labelling in the marmoset OSE cells at early, mid and late stages of the follicular phase

Animal	Early Follicular		Mid follicular		Late follicular	
	Stained	Not-Stained	Stained	Not stained	Stained	Not Stained
1	3	1979	5	1847	9	1861
1	6	2094	2	1795	7	1814
2	2	1235	8	1864	6	1389
2	3	1634	9	1811	11	1347
3	0	1928	5	1310	4	1789
3	2	875	1	1877	5	1157
4	4	1522	6	1461	17	1831
4	2	1394	2	1219	4	926
5			8	1878	10	1841
5			5	1413	6	931
6					5	1297
6					11	1101

Table 5.2

BrdU labelling in the marmoset OSE cells at early, mid and late stages of the luteal phase

Animal	Early luteal		Mid luteal		Late luteal	
	Stained	Not-Stained	Stained	Not stained	Stained	Not Stained
1	7	1391	7	1461	2	1507
1	4	1678	4	1349	3	1747
2	19	1126	19	1024	2	993
2	2	1812	2	1329	2	996
3	5	886	5	1216	1	1357
3	12	1326	12	951	3	914
4	9	1450	9	852	0	1192
4	2	847	2	913	1	1711
5	10	1327	10	1285	0	865
5	8	1324	8	974	2	1425

Table 5.3

BrdU labelling in the marmoset OSE cells at week-1, 2 and3 pregnancy

Animal	Pregnant (week-1)		(Pregnant week-2)		Pregnant (week-3)	
	Stained	Not-Stained	Stained	Not stained	Stained	Not Stained
1	0	967	1	901	0	947
1	5	893	0	897	0	1002
2	0	900	0	996	0	989
2	4	875	0	781	0	1103
3	1	1341	0	964	0	997
3	9	1433	1	835	0	978
4	1	911			0	1322
4	1	1215			0	1014
5	0	1769			0	812
5	2	992			0	974
6					0	1016
6					0	932

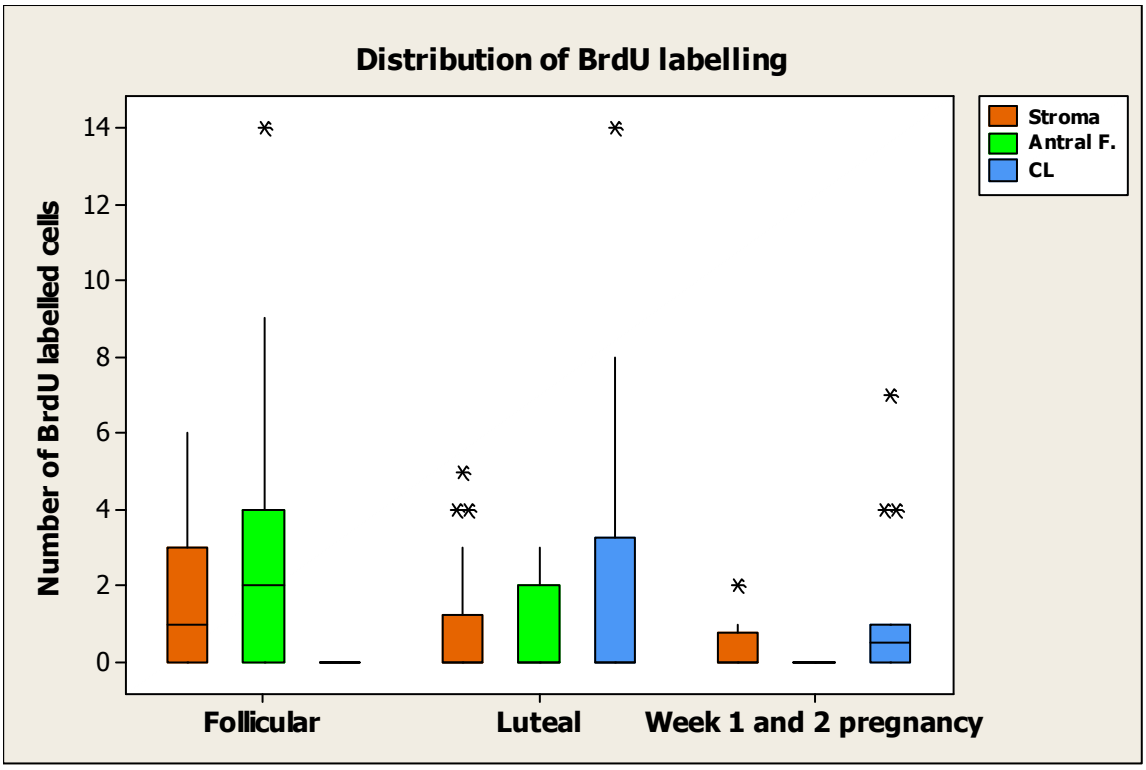


Figure 5.3 Box plots indicating the distribution of BrdU labelled cells in marmoset OSE through different regions (overlying stroma, antral follicles and corpora lutea (CL)) at the follicular phase (n = 15), the luteal phase (n = 15) and at week 1 and 2 pregnancy (n = 8). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Data are the number of BrdU labelled cells in OSE cells at each region for each section (2 sections/ animal). Total number of counted cells is presented in Table 5.4, 5.5 and 5.6.

Table 5.4

The distribution of BrdU labelled cells in the marmoset OSE cells over different regions of the ovary at the follicular phase

Group	Animal	CL	GF	Stroma	Total
Early follicular	1	0	0	3	3
	1	0	2	4	6
	2	0	2	0	2
	2	0	2	1	3
	3	0	0	0	0
	3	0	2	0	2
	4	0	3	1	4
	4	0	2	0	2
Mid follicular	1	0	1	4	5
	1	0	2	0	2
	2	0	2	6	8
	2	0	4	5	9
	3	0	4	1	5
	3	0	0	1	1
	4	0	1	6	6
	4	0	0	2	2
	5	0	2	6	8
	5	0	2	3	5
Late follicular	1	0	9	0	9
	1	0	7	0	7
	2	0	5	1	6
	2	0	9	2	11
	3	0	2	2	4
	3	0	5	0	5
	4	0	14	3	17
	4	0	4	0	4
	5	0	9	1	10
	5	0	6	0	6
	6	0	5	0	5
	6	0	9	2	11
Total		**0**	**115**	**54**	**169**

Table 5.5

The distribution of BrdU labelled cells in the marmoset OSE cells over different regions of the ovary at the luteal phase

Group	Animal	CL	GF	Stroma	Total
Early luteal	1	5	0	2	7
	1	4	0	0	4
	2	14	0	5	19
	2	1	0	1	2
	3	3	0	2	5
	3	8	0	4	12
	4	5	0	4	9
	4	2	0	0	2
	5	7	0	3	10
	5	7	0	1	8
Mid luteal	1	0	0	1	1
	1	0	0	2	2
	2	0	0	1	1
	2	0	0	0	0
	3	0	2	0	2
	3	0	0	1	1
	4	1	0	0	1
	4	0	0	0	0
	5	0	0	0	0
	5	0	2	0	2
Late luteal	1	0	2	0	2
	1	0	3	0	3
	2	0	2	0	2
	2	0	2	0	2
	3	0	1	0	1
	3	0	3	0	3
	4	0	0	0	0
	4	0	1	0	1
	5	0	0	0	0
	5	0	2	0	2
Total		**57**	**20**	**27**	**104**

Table 5.6

The distribution of BrdU labelled cells in the marmoset OSE cells over different regions of the ovary at week-1 and week-2 pregnancy

Group	Animal	CL	GF	Stroma	Total
Pregnant Week-1	1	0	0	0	0
	1	4	0	1	5
	2	0	0	0	0
	2	4	0	0	4
	3	1	0	0	1
	3	7	0	2	9
	4	1	0	0	1
	4	0	0	1	1
	5	0	0	0	0
	5	1	0	1	2
Pregnant Week-2	1	1	0	0	1
	1	0	0	0	0
	2	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	3	1	0	0	1
Total		**20**	**0**	**5**	25

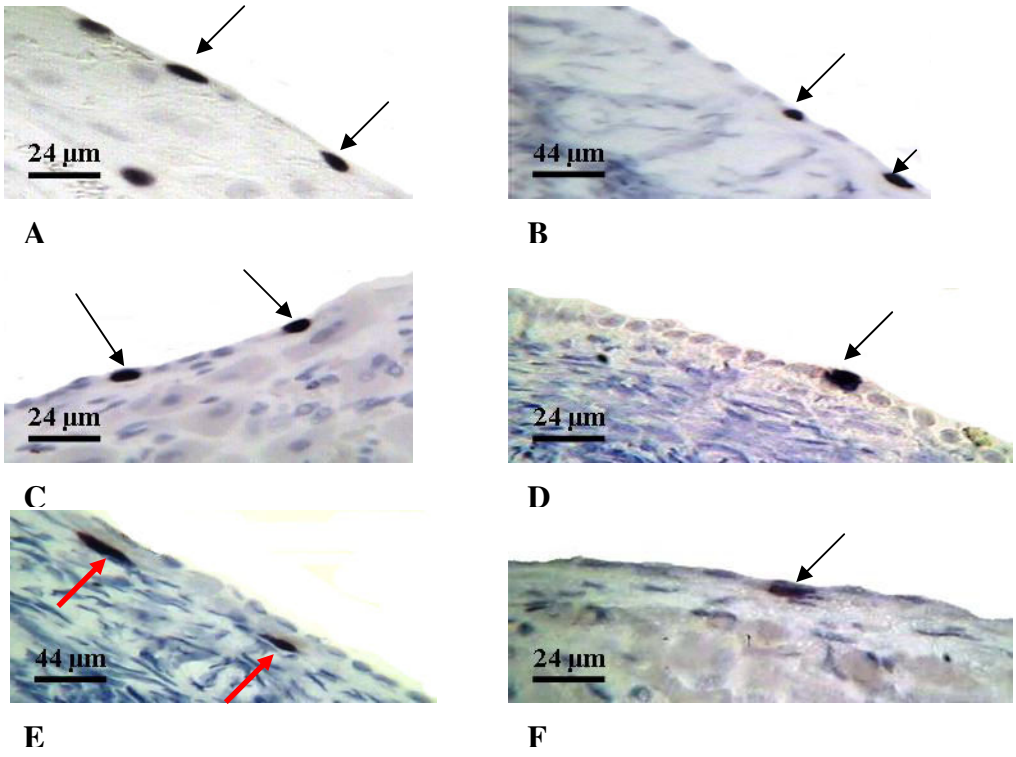


Figure 5.4 Photomicrographs showing BrdU labelling within the marmoset OSE cells. BrdU labelling within the OSE cells at follicular phase of the ovulatory cycle (A) over large antral follicle and (B) over stroma. C-D demonstrate the morphology of OSE cells at luteal phase (C) over mature CL and (D) over stroma. E-F OSE cells at pregnancy (E) over stroma and (F) over CL. Red arrows indicate labelled cells within the tunica albuginea layer (black stain).

5.3.2 Morphological changes in the OSE cells

The distribution of proliferation markers in OSE cells varied considerably during different reproductive stages (follicular, luteal and pregnancy). The OSE morphology and its adhesiveness to underlying tissues are also expected to alter considering the extent of multiplication capabilities within a limited surface area in which the cells can expand. The microscopic observation revealed considerable variation in the shape of the OSE cells ranging from squamous to cuboidal. Moreover, the association between the OSE cells was affected by the ovarian cycle and the ovarian compartments. OSE cells over the large antral follicles which were located very close to surface were flattened in shape and disarrangement between these cells was noticed. There was no evidence of firm attachment with the underlying basement membrane (Figure 5.5-A).Over the CL, OSE cells appeared as squamous with very weak association between the cells, and it was seen that attachment of these cells with the basement membrane was also loose (Figure 5.5-B). The OSE cells observed in a region away from the follicular development and the ovarian event over the stroma, displayed highly organised arrangement of cuboidal shape, and attachment in between the cells and of the cells with underlying basement membrane was also firm (Figure 5.5-C)

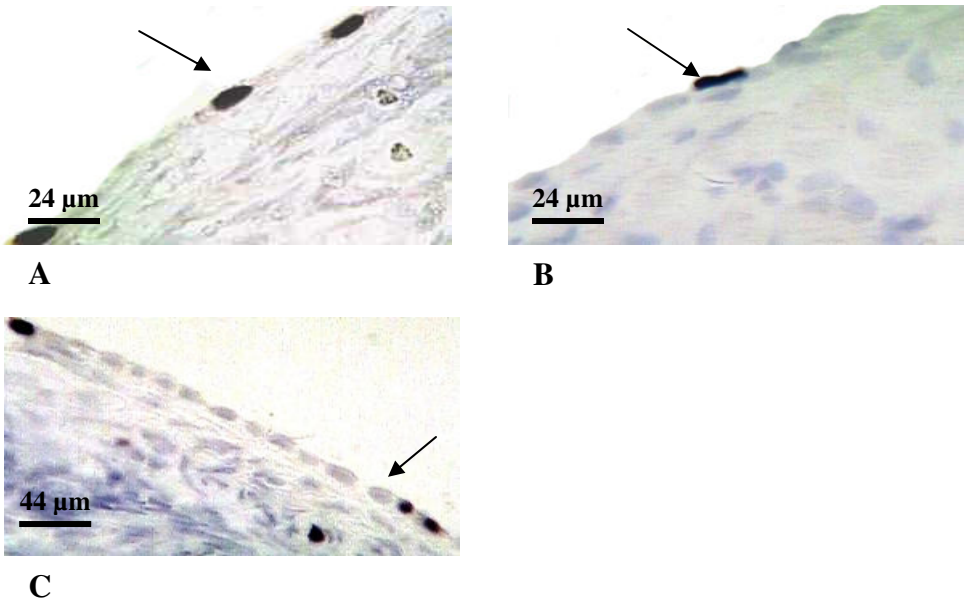


Figure 5.5 Photomicrographs representing the morphological changes of the OSE cells at different regions of the marmoset ovary. (A) OSE covering large antral follicle (arrow indicates flattened shaped cells), (B) over a mature CL (arrow indicates squamous shaped cells) and (C) over ovarian stroma (arrow indicates cuboidal shaped cells).

5.3.3 Effect of Antarelix treatment on OSE proliferation

Prior studies on Antarelix treatment in marmoset monkeys revealed significant reduction in BrdU labelling in ovarian endothelial cells and steroidogenesis in granulosa and theca cells of growing follicles (Taylor *et al.* 2004). It was a matter of interest to study how these changes would affect the overlaying OSE cells. A comparison of sections prepared from the ovaries of late follicular controls and the treatment groups revealed that the OSE cells of GnRH antagonist-treated ovaries from day 0-10 and from day 5-10 exhibited a decrease in the proliferation after both treatment schedules. Quantitative analysis confirmed significant decrease in cell proliferation in the OSE cells in both treated groups, and the effect was more pronounced in the longer treatment schedule (day 0-10 treated group; $P = 0.001$) (Figure 5.6)

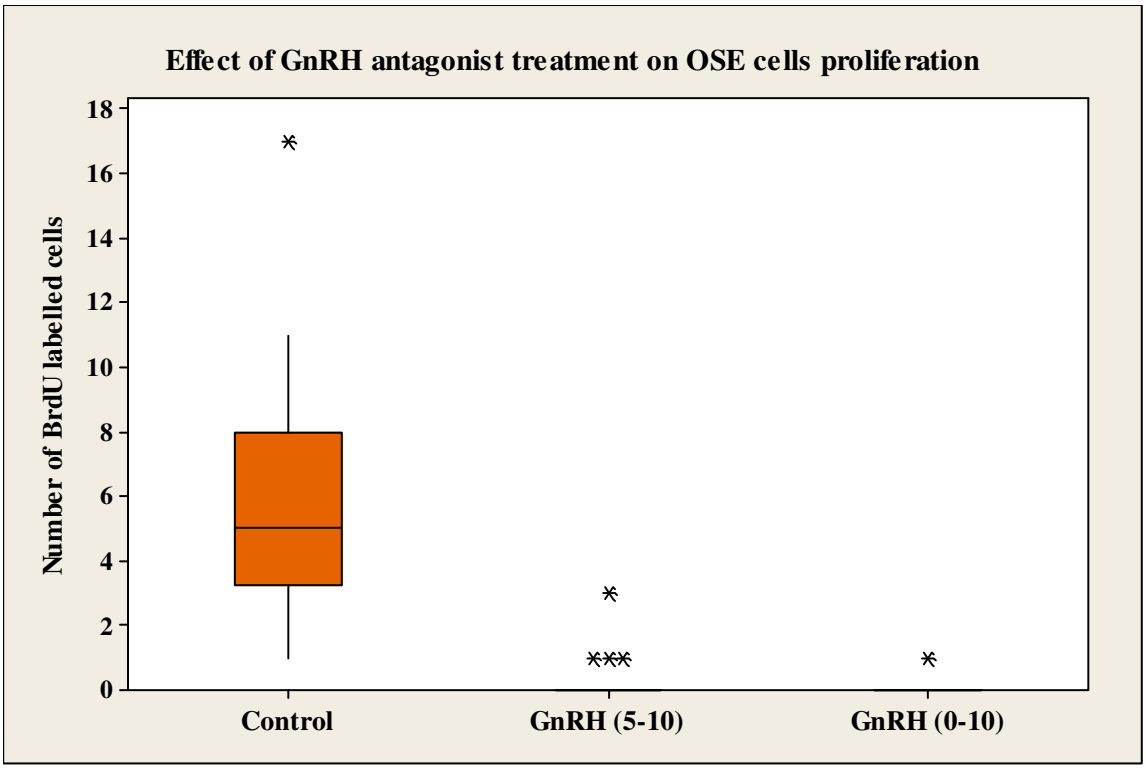


Figure 5.6 Box plots indicating BrdU incorporation into the marmoset OSE cells over the total surface area of the ovary after GnRH-antagonist treatment from day 5-10 (n = 5; 5 sections/animal) and day 0-10 (n = 5; 3 sections/animal) follicular phase, ovaries from day 10 follicular phase were used as a control (n = 5; 4 sections/animal). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Data are the number of BrdU labelled cells in OSE cells for each section. Total number of counted cells is presented in Table 5.7.

Table 5.7

BrdU labelling in the marmoset OSE cells over the total surface area of the ovary after GnRH antagonist treatment

Control			GnRH-antag 5-10			GnRH-antag 0-10		
Animal	**No. labelled cells**	**No. unlabelled cells**	**Animal**	**No. labelled cells**	**No. unlabelled cells**	**Animal**	**No. labelled cells**	**No. unlabelled cells**
1	5	1847	1	1	1865	1	1	1425
1	2	1795	1	0	1998	1	0	1491
1	8	1864	1	0	1975	1	0	1502
1	9	1811	1	0	1774	2	0	1479
2	5	1310	1	1	1876	2	0	1568
2	1	1877	2	0	1943	2	0	1801
2	8	1862	2	0	1612	3	0	1799
2	7	1814	2	0	1787	3	0	1788
3	6	1389	2	1	1914	3	0	1695
3	11	1347	2	0	1783	4	0	1702
3	4	1789	3	0	1881	4	0	1429
3	5	1157	3	0	1679	4	0	1473
4	17	1831	3	0	1862	5	0	1398
4	4	926	3	0	1993	5	0	1412
4	10	1841	3	0	1765	5	0	1463
4	3	934	4	0	1871			
5	5	1297	4	0	1840			
5	6	1106	4	0	1895			
5	3	1979	4	0	1882			
5	3	2094	4	0	1612			
			5	3	1792			
			5	0	1747			
			5	0	1913			
			5	0	1862			
			5	0	1522			

5.4 Discussion

Considering the fundamental role of OSE cells in ovarian tumourigenesis, the growth regulation of normal and neoplastic OSE cells by intraovarian regulators may play an important role in ovarian cancer development. In this regard, GnRH has been implicated as an autocrine regulator of normal OSE (Kang *et al.* 2000) as well as a growth inhibitor of ovarian cancer cells. GnRH antagonists are widely used in the clinic during ovarian hyperstimulation programmes to block endogenous gonadotrophin secretion (Huirne *et al.* 2004). GnRH regulates the biosynthesis and secretion of gonadotrophins. In this study we have demonstrated two significant findings regarding the OSE cell proliferation, under the effects of pregnancy (Page 104) and in response of GnRH antagonist treatment (Page 111). We provide evidence that GnRH antagonist treatment and pregnancy both significantly reduce the proliferative activity of the OSE.

The present results show that during the ovulatory cycle, BrdU staining of OSE layer increases towards end of the follicular phase, when it is in contact with large antral follicles, and decreases by the mid luteal phase. In fact the highest staining was seen in CL at early luteal phase. Low staining of OSE, overlying the stroma shows that the resting OSE cells at early follicle development or those which are not in the vicinity of the CL or antrum are not rapidly dividing. Since OSE proliferation was associated with large antral follicles and CL at all reproductive phases it can be predicted that events leading to luteogenesis contribute towards OSE proliferation whereas the reverse process, luteolysis, suppresses the proliferative response. Before luteogenesis remarkable changes take place in the granulosa and theca cells of growing follicles in late follicular phase, notably vascularization, angiogenesis and steroidogenesis. Interestingly, these changes are regulated by LH (Dickson & Fraser 2000). Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor that plays an important role in angiogenesis (Redmer *et al.* 2001). In granulosa cells of growing follicles, VEGF expression is up-regulated most likely due to LH, and high cellular level of this factor is maintained up to luteal phase controlled by LH/CG (Stouffer *et al.* 2001). In the CL the same granulosa cells transform to granulosa-lutein cells responsible for steroidogenesis. It was therefore a matter of interest to examine whether or not the angiogenic and

steroidogenic granulosa cells proliferate at late follicular and early luteal phase. The granulosa cells of growing follicles were heavily stained suggesting that these are highly proliferative cells, and these rapidly multiplying granulosa cells may secrete some mitogenic substances, notably β-oestradiol, to influence the surrounding OSE layer to proliferate.

OSE cell morphology is believed to be another marker of proliferative activity (Auersperg *et al.* 2001). It has been suggested that squamous and cuboidal forms of cells represent the groups that have not or have undergone post-ovulatory proliferation respectively (Gillett *et al*. 1991). OSE cells tend to assume columnar shapes when they form clefts and inclusion cysts (Auersperg *et al.* 2001). These cells are the primary targets for mitogenic and inflammatory action leading to tumourigenic growth (Deligdisch *et al.* 1995). The OSE cells attach to the basement membrane that participate in a number of fundamental biological processes such as cell growth, differentiation tissue development and repair (Couchman & Woods 1993; Aumailley & Krieg 1996; Aumailley & Gayraud 1998). It has been proposed that loss of ovarian surface basement membrane after preovulatory stimulation by gonadotrophins is a critical step in early tumourigenicity of the OSE (Yang *et al.* 2002; Roland *et al.* 2003). We found that non-proliferative cuboidal and tightly attached OSE cells were present away from the ovulation sites. The proliferative squamous cells with very loose attachment were discernable over CL and disarranged flattened cells were present in the OSE layer just before ovulation. Some of these cells might transform to columnar cells if inclusion cysts or clefts are formed after ovulation. Interestingly, the stratification feature which was common in the OSE layer of pregnant ewes but did not occur in marmoset, an explanation of this event would be seasonality in sheep. During anoestrous and due to anovulation cycle, the OSE cells enter into a quiescence state (no excessive injury and repair) (less chance of cells slough off) this may lead to accumulation of cells in multiple layers.

Although it was not expected to detect OSE proliferation during pregnancy, OSE cells during 2nd week of gestation displayed positive BrdU staining specifically in proximity to CL. These observations suggest that the OSE cells at this stage are still under the influences of the CL mitogenic factors or probably because the progesterone concentration has not been high enough to suppress any proliferative action. Earlier we

demonstrated an anti-proliferative role of progesterone, whether produced during pregnancy or given as contraceptive pills. It is reasonable to assume that despite progesterone presence, CL influences the OSE proliferation and this attribute could be due to some factor which supersedes the hormone action. Kisliouk and colleagues (2005) indicated the role of prokineticins-1 (PK-1), a member of secreted proteins family protineticins in bovine corpora lutea. It has been shown that PK-1 acts as a mitogenic and antiapoptotic factor in luteal function (Kisliouk *et al*. 2005). The data also suggests that as pregnancy advances the proliferation becomes diminished because of the increasing levels of progesterone.

So far the results indicate that, in marmosets, luteogenesis rather than folliculogenesis seems to influence OSE proliferation and luteolysis and pregnancy reverses this process. In primates LH/CG is necessary for CL development, which induces numerous paracrine regulators of luteal development including VEGF that controls angiogenesis and vascularization (Duncan & Kalluri 2009; Fraser & Duncan 2009). As LH concentration starts to recede, the natural luteolysis advances starting from mid luteal phase. During luteolysis the LH level falls and all its stimulatory actions like angiogenesis and steroidogenesis decline. Additionally, luteal cells produce interleukins, prostaglandins and factors like capsase-3 to induce apoptosis and other mechanisms to degenerate CL (Fraser *et al.* 2006). If CL is rescued due to pregnancy, CG produced from conceptus takes over the LH functions. It remains to be seen whether in primates local ovarian environment influences OSE proliferation or it is simply the mechanical ablation of OSE layer to accommodate large antrum or CL that triggers the renewed multiplication and neoplastic transformation. In this context it is noteworthy that in rhesus monkeys brushing the OSE layer at any reproductive phase is sufficient to induce proliferative repair (Wright *et al.* 2008).

In this investigation, the potential anti-proliferative response to treatment with a GnRH antagonist was examined on OSE cells in marmoset monkeys. Prior to this experiment luteolysis of the CL was artificially induced and the luteal phase was prematurely stopped by treatment with prostaglandin $F_{2\alpha}$ analogue towards the end of the ovulatory cycle, which is a common practice to synchronize follicular phase in cycling marmosets (Steinetz *et al.* 1995). Luteolysis was verified as a sharp reduction in

serum progesterone concentration (Taylor *et al.* 2004). Towards the end of luteolysis the steroidal hormones reach their minimum concentrations and under FSH and LH/CG influence the steroidogenesis begins in pre-antral granulosa and antral granulosa and theca cells. Blocking FSH and LH/CG release at zero time by Antarelix (0-10 days) in this study would have arrested the development of tertiory follicles with consequent prevention of ovulation (Taylor *et al.* 2004). Further, β-oestradiol synthesis was decreased as Antarelix treatment inhibits steroidogenesis (Taylor *et al*. 2004). At 5th day blockage (5-10 days) some gonadotrophin concentration would have build up in within first 5 days which apparently is sufficient to grow follicles up to pre-antral stage. Thereafter, LH "surge" which takes place at late follicular phase necessary for ovulation and CL formation must have been prevented. Since in marmosets large antral follicles are differentiated only after 6 days (Gilchrist *et al.* 2001), in this situation only the small antral follicles are expected to develop and then start to regress into atresia (Taylor *et al.* 2004). The depressive effect of Antarelix on OSE proliferative activity was clearly discernable regardless of the time of administration. It is proposed that pre-ovulatory large antral follicle and its derived CL may be responsible for OSE proliferation in the contact regions. This conclusion is derived from the observation that OSE proliferation was suppressed when drug was administered on 5th day of follicular cycle when pre-ovulatory follicles were not able to be differentiated. There could be several explanations of Antarelix's OSE anti-proliferative effect which may function in isolation or in combination: 1) reversal of LH/CG up-regulation of localized growth factors like IGF and HGF which are responsible for mitogenic action through their anti-apoptotic effect on OSE, 2) inhibition of gonadotrophin-induced steroidogenesis in antral follicles and CL would minimize production of mitogenic β-oestradiol in granulosa cells, and consequently OSE proliferation would be inhibited, 3) GnRH antagonists result in skew in frequency of antral follicles (Taylor *et al.* 2004), and apparently this would reduce ovulation frequencies and resultant epithelial rupture, and thereby prevent the neoplastic growth of OSE, and 4) this compound may bind to GnRH-I/ GnRH-II receptors in OSE cells itself and inhibit all downstream pathways which involve mitogen activating protein (MAP), P38 and the extracellular signal regulate kinase (ERK-1/2) responsible for mitogenic proliferation (Millar *et al.* 2001; Mamputha *et al.* 2007).

In conclusion, BrdU uptake and immuoreactivity equating to proliferating cells was detected at the ovulation site before and after ovulation and lower number of BrdU–stained cells were found to be distributed randomly in OSE at other sites around the ovary, suggesting that the OSE proliferative activity takes place in a cyclic manner depending on the ovarian events that occur underlying the OSE during the ovarian cycle. GnRH antagonist treatment resulted in complete inhibition of OSE cells proliferation. Further study is needed to investigate the mechanisms in which the GnRH antagonist exerts its effect on OSE cells, whether by direct or indirect pathways.

CHAPTER SIX

General Discussion

6.1 General discussion

The work presented in this thesis demonstrates how OSE cells are influenced by many factors intrinsic and extrinsic to the ovary. The ovary is a dynamic organ with constantly changing structures and micro-environments. The entire ovary is covered by the OSE layer which is believed to be the progenitor of most EOC (Auersperg *et al.* 1998); the most lethal type of cancer in women. Most established risk factors for this disease relate to reproductive events (Tung *et al.* 2005; Murdoch 2005; Murdoch *et al.* 2005). Malignant transformation of normal ovarian epithelial cells is caused by genetic alterations that disrupt regulation of proliferation, programmed cell death and senescence. The rate of proliferation is a major determinant of the number of cells in the population and to prevent excessive proliferation, DNA synthesis and cell division are normally controlled within the cell cycle. There is general agreement that the risk of ovarian cancer decreases with increasing parity and longer duration of oral contraceptive use (Whittemore *et al.* 1992a; Negri *et al.* 1991). The main aims of this thesis were 1) to examine the proliferative activity of the ovine OSE cells through the regular ovarian cycle and during pregnancy by monitoring proliferation *in vivo* using IHC for PCNA and Ki-67, and to investigate whether the underlying ovarian structure specifically growing follicles have any influences on the OSE layer; 2) to examine the role of growing follicles and corpora lutea and their contents of steroids and growth factors in the regulation of OSE proliferative activity by using cultured OSE cells; 3) to test the hypothesis that pregnancy may reduce EOC risk by inducing apoptosis within the OSE layer and inclusion cysts, a process mediated through over-expression of p53, which is under the control of high progesterone and oestrogen level; and 4) to examine the role of pregnancy and gonadotrophins inhibition (administration of GnRH antagonist) on OSE cells morphology and proliferation in a non-human primate, the marmoset monkey, by using the IHC method for BrdU at different stages of the cycle and during pregnancy.

The investigations carried out in this thesis have revealed that pregnancy inhibits epithelial proliferation and this may be responsible for a reduction in the incidence of EOC, either by inhibiting the production of gonadotrophins and concomitant localized

growth factors and/or by enhancing the p53-mediated apoptosis of damaged and mitogenic cells arising from repeated ovulations.

During the reproductive cycle (cycling, anoestrus and pregnancy) major tissue modulation and reorganization of ovarian epithelium takes place and this is under tight regulation of gondotropins, localized growth factors and steroidal hormones. All of these factors will influence OSE, but the OSE is not homogeneous, as different areas will be subjected to different factors depending on the underlying tissue. The *in vivo* interaction between the OSE cells and the underlying compartments, specifically the growing follicles, is poorly understood.

In chapter 2 the relationship between the stage of the reproductive cycle, follicle dynamics and OSE activity was investigated, and consistent with a general notion, pregnancy did not affect activation of the earliest stages of follicle development (primordial), though it suppressed the later stages of development and differentiation. The proliferative activity of granulosa cells within growing follicles decreased during pregnancy relative to the non-pregnant animals, and this may represent altered growth rates at later stages of development, i.e. follicles grow more slowly during pregnancy. Differences in circulating gonadotrophins and steroidal hormones are the likely causes of follicle suppression and steroidogenesis in granulosa cells. Pituitary gonadotrophins, FSH and LH levels are low during pregnancy (Taya & Greenwald 1981b; Taya & Greenwald 1981a). FSH has been reported to accelerate granulosa cell proliferation and differentiation in cows (Hulshof *et al.* 1994), sheep (Newton *et al.* 1999) and humans (Roy & Treacy 1993). In agreement with our results, in golden hamsters (Greenwald *et al.* 1967) and cows (Guilbault *et al.* 1986) during late pregnancy, a pool of transitory follicles was observed, whereas the large follicles were missing as they had degenerated. High levels of progesterone might be involved in attenuation of follicular development. Progesterone inhibits gonadotrophin induced follicular growth in hypophysectomised rats (Fukuda *et al.* 1980), and also inhibits the induction of aromatase activity in rat granulosa cells responsible for steroid production (Fortune & Vincent 1983). Loss of follicular oestrogenic activity could be responsible for suppression of follicular development. Besides, it has been suggested that the conceptus exerts follicle

suppressive effects in pregnant ewes (al Gubory & Abdennebi 1996), especially the development of the antral follicle. Moreover, residual local inhibitory effects of CL on folliculogenesis in pregnant cows may reduce the number of antral follicles (Bellin *et al.* 1984).

Using IHC and cell proliferation markers (PCNA, Ki-67 and BrdU), we have demonstrated that pregnancy not only affected the proliferation of granulosa cells but also reduced the proliferative activity of OSE cells during early and late stages of pregnancy (sheep and marmoset monkeys). We propose that suppression of OSE cells' proliferative activity due to pregnancy is a consequence of prolonged exposure to high levels of progesterone, accompanied by low levels of gonadotrophin. Over the first month of pregnancy, maternal LH and FSH decline and trophoblast hCG increases, which stimulates the CL to continue producing progesterone and not to regress (Jaffe 1991; Yen 1994). A direct apoptotic effect of progesterone on the OSE cells was demonstrated in cell cultures of OSE from bovine ovaries (Chapter 4). Consistent with our findings, it was indicated that progesterone treatment *in vitro* inhibits the growth of immortalized normal and malignant human OSE cells through apoptosis induction (Syed & Ho 2003).

A popular explanation for the onco-protective effect of pregnancy is temporary cessation of ovulation (Fathalla 1971). This would reduce constant ovarian epithelial disruption and repair that may induce ovarian neoplasia, possibly through the accumulation of mutations in tumour suppressor genes such as *p53* (Schildkraut *et al.* 1997). The results with heifers in chapter 4 demonstrate that progesterone induces apoptosis in cultured bovine OSE. Moreover, the *in vivo* observations revealed that pregnancy led to high apoptotic index in OSE cells and in cells lining inclusion cysts. In contrast, non-pregnant animals did not produce any evidence of apoptosis in either OSE or inclusion cysts. Apparently, elevated progesterone during pregnancy may play a role in regulating programmed cell death in OSE. Consistent with our finding, Rodriguez *et al.* (1998) demonstrated that *in vivo* administration of progesterone to the OSE cells of monkeys induced apoptosis. The protective role of progesterone during pregnancy could

be the result of a clearing process or exfoliation from the ovary (particularly the OSE layer and inclusion cysts) of any DNA damaged OSE that might lead to malignancy.

Apoptosis may be a key factor to avert such malignant transformation, by way of elimination of the cells that have undergone mutations. Progesterone may also up-regulate the tumour suppressor gene *p53*, which is a critical regulator of cell cycle arrest and apoptosis in response to DNA damage. Mutation of the *p53* tumour suppressor gene is the most frequent genetic lesion described in human cancers (Berchuck *et al.* 1994), and it is known to play a pivotal role in regulation of both proliferation and apoptosis (Braithwaite *et al.* 1987; Rotter *et al.* 1983). In normal cells, p53 protein exerts its tumour suppressor activity by binding to transcriptional regulatory elements of the genes that act to arrest cells at G1 phase. Additionally, p53 is thought to play a role in preventing cancer by stimulating apoptosis of cells that have undergone excessive genetic damage (Kuerbitz *et al.* 1992). In this regard, p53 has been described as the "guardian of the genome" since it delays entry of cells into S phase until the genome has been cleansed of mutations (Lane 1992). If DNA repair is inadequate, p53 may initiate apoptosis, thereby eliminating the cells with altered or damaged genotype. In this study, it was shown that p53 was more highly expressed within OSE cells overlying the CL, with less expression in those overlying the large antral follicles, which might be the preovulatory follicle (Chapter 4). In contrast, p53 protein was completely absent within the OSE cells in those areas furthest from ovulation events. Moreover, the *in vitro* results demonstrate that progesterone administration significantly increased p53 expression in bovine OSE cells. Expression of p53 within OSE cells over the CL may contribute to the re-epithelization process. P53 might arrest the cell cycle, enabling DNA repair before replication, or it might induce apoptosis if DNA damage is irreparable. Therefore, besides cessation of ovulation, pregnancy is also associated with elevated progesterone and this may have a protective role by inducing p53 expression.

High risk of ovarian cancer is almost invariably associated with elevated gonadotrophin secretion such as at the postmenopausal stage and after receiving ovulation induction drugs (Rao & Slotman 1991; Whittemore *et al.* 1992b; Shoham 1994). In marmoset monkeys treated with GnRH antagonist, OSE proliferation was

found to be markedly suppressed (Chapter 5). This finding is consistent with the theory that pregnancy protection role is a combination of low gonadotrophin as well as high progesterone. Histological studies indicate that the growth and function of OSE cells is regulated by paracrine and/or endocrine pathways (Parrott *et al.* 2000b; Nilsson *et al.* 2001a). OSE is an avascular tissue (Clement 1987), therefore a predominant influence of paracrine rather than endocrine factors is expected. Both epidemiological and experimental investigations have implicated sex steroids in the pathogenesis and growth regulation of ovarian cancer (Lukanova *et al.* 2003). Most of the ovarian steroidogenesis occurs in the granulosa and theca cells of developing and mature follicles under the control of the gonadotrophins, FSH and LH (Howles 2000; Hillier 2001). Oestrogen biosynthesis peaks sharply in the granulosa cells prior to ovulation (Agca *et al.* 2006; Murdoch & Van Kirk 2001). As follicular growth distends the surface of the ovary, the epithelial cells multiply and become flattened in shape (Gillett *et al.* 1991) until ovulation, when the epithelial cell's proteases dissolve the follicle apex and rupture it (Kruk *et al.* 1994). It has been suggested that the epithelium during this stage is exposed to more paracrine influences from the granulosa and theca cells (Hafez *et al.* 1980), or this is mediated through diffusion of follicular fluid (Carcangiu & Chambers 1992), as before ovulation this fluid may contain high concentration of oestradiol (Murdoch & Van Kirk 2001). Results from chapter 3 show that *in vitro* administration of oestrogen and progesterone did not stimulate OSE proliferation, suggesting neither of the steroids affect cultured ovine OSE cells, whereas, IGF-1 significantly induced proliferation. Moreover, observed stimulatory effect of the follicular fluid treatment may be related to ovarian factors other than steroids, such as EGF and IGF-1.

In the present study, immunohistochemistry data (PCNA, Ki-67 and BrdU) indicate that the proliferative activity of OSE cells is related to the ovulation and post-ovulation repair process. Although immunohistochemistry results in chapters 2 and 5 showed a variation in the percentage of immunureactivity within OSE cells, the overall proliferative indexes were very low compared to the other ovarian compartments such as granulosa cells. It seems that during ovarian cycles, the epithelium proliferates at times when mitogenic influences are relatively greater, and increased mitotic activity is likely

to enhance the risk of mutations. This genetic aberration could then accumulate with additional epithelial cell division in further cycles during re-epithelization.

In this study, some results were different from expected, and further study is required to elucidate these issues. Firstly, there was marker-based difference in recognition of the proliferative activity among different cell types, i.e. epithelial cells, granulosa cells and oocytes (Chapters 2 and 5). In cycling ewes, a baseline PCNA staining in granulosa cells of all follicular stages was consistently observed. In contrast, the Ki-67 marker was restricted to only the late follicles. One explanation may be that DNA damage and repair activity takes place even in non-dividing primordial follicles, possibly due to pre-granulosa cell formation and accommodation of oocytes. Correspondingly, PCNA staining was discernable in oocytes, suggesting that high turnover of DNA in these cells might create an environment for the surrounding cells to undergo DNA modulation. Thus, despite Ki-67 negativity we cannot entirely rule out that the primordial-transitory follicles are non-proliferative. In marmoset monkeys, low but significant BrdU staining of "resting" OSE over stroma was seen, which indicates that these cells are also dividing, albeit slowly. This study hypothesizes that this discrete proliferation accounts for the formation of the stem cells.

Secondly, the OSE proliferation during early-pregnancy was not expected in marmosets, especially over CL (Chapter 5), whereas progesterone concentration must have been high enough to suppress this proliferation. It appears that pro-proliferative factors originating from CL might supersede the progesterone's action, and this effect may extend to early pregnancy. The nature of this factor is not known.

Thirdly, in heifers during mid-pregnancy the number of inclusion cysts increased and epithelial cells of these cysts and OSE exhibited substantial apoptotic index revealing extensive cell death (Chapter 4). Elevated P53 expression in these cells corroborated the fact that apoptotic activity was indeed high. In the normal course of events, formation of inclusion cysts and epithelial apoptosis is confined to the ovulation process. It is reasonable to assume that at mid-pregnancy, due to some unknown factors, there is a tendency of neoplastic transformation of OSE cells and high apoptosis stimulated by progesterone is a mechanism to avert this.

6.2 Concluding remarks

The work presented here has used different model systems to examine and characterise OSE under different conditions. This comparative approach using three animal models, different timings through the ovarian cycle and different cell proliferation markers has attempted to elucidate the possible mechanisms by which pregnancy affects the OSE. It is concluded that the possible protective role of pregnancy on progression of ovarian cancer is at least in part related to an inhibition of OSE cell proliferation and accelerated cell death. Ovarian follicles continue to grow throughout pregnancy but they do not reach the ovulatory stage, therefore, cyclic proliferative activity of the surface epithelium (OSE injury and repair) is suppressed and a quiescent state is maintained. We provide evidence to support an involvement of progesterone in apoptosis induction and cell growth inhibition in OSE cells. Additionally, data from study of the *p53* tumour suppressor gene indicates that this gene, whose expression is governed by progesterone and/or some other hitherto unknown factors, might play an important role in onco-protection. This feature saves the genome from progressive accumulation of mutations. We suggest that any defect in the p53 pathway may lead to malignancy either by mutation in the *p53* gene, and/or by down-regulation of a cascade of reactions responsible for cell cycle attenuation and apoptosis. Expression of p53 has to be dealt with care as this protein has a short-half life, and this may give false negative labelling and erroneous results.

The choice of different animal models in this study reflects differences in ovulation rate and seasonality i.e. cyclical activity, all of which may affect proliferation rate i.e. ewes (mono-ovular seasonal breeder), cows (low ovulation rate) and marmoset monkeys (poly-ovular). Ovine ovaries provided information on OSE during the anoestrus or resting period from ovulation. Our results indicate that OSE cells were in a quiescent state and this could explain why ovarian cancer does not occur in these animals. Cows turned out to be good models but there were experimental limitations: the animals brought from local slaughterhouse were young and it was too early to detect any defect or malformation within OSE or inclusion cysts. Moreover, the age or parity of

these animals was not known. If older animals were studied it would have been possible to analyze the etiology of ovarian cancer in the quiescence state.

Overall, the picture from the *in vitro* supplementation of hormones/growth factors on induction of apoptosis or stimulation of proliferation did not entirely match with the corresponding analysis on OSE layer in the *in vivo* models. For example, progesterone had practically no influence over the cultured sheep OSE cell proliferation, but high progesterone level during pregnancy remarkably suppressed the OSE cell proliferation within the ovary. Apoptosis induction by progesterone was clearly discernable in heifer OSE cultured cells but was not observed in late pregnancy, at which stage progesterone level should have been high. It is likely that absence of the TA layer in the *in vitro* study may have altered the physiological response of OSE cells to different treatments. Cultured OSE are grown in a different environment and are exposure directly to the effectors in the medium, whereas inside the ovary TA may selectively hinder exposure of OSE cells to the effectors produced within stroma or follicles, or coming from the circulation. The exact influence of such effectors can be better judged in the epithelium of inclusion cysts which are situated within the ovarian stroma, and are more exposed to the compounds. Hence, besides searching for a suitable animal model for studying OSE physiology, we need to develop a unique OSE culture system that mimics the conditions prevailing *in vivo*. For these kinds of basic investigations human subjects cannot be used, therefore it is important to identify suitable animal and *in vitro* models. Given that the work presented in this thesis shows the effect of underlying follicular structures on OSE characteristics it can be concluded that the isolated OSE monolayer culture model may not be the most appropriate. To study human OSE in more detail it would be beneficial to have a culture model that supports OSE and underlying follicular tissue *in vitro*. A culture model recently developed in our laboratory supports the growth of follicles and overlying OSE cells (Telfer & McLaughlin, 2007, Telfer *et al.* 2008). The main aim of this system is to support follicle development but with adaptation it could be utilised to study the OSE interactions with defined follicular components. Such artificial systems would facilitate

mimicking the effect of follicular cycle and holds promise for future research on human OSE cells.

6.3 Future perspective

The work presented in this thesis has highlighted many gaps in our understanding of OSE cells. An understanding of their basic characteristics is fundamental to advancing our knowledge of the causes of aberrant development. Efforts have been made over the years to understand the biology of the surface epithelium in an attempt to explain the tendency of these relatively inconspicuous ovarian cells to undergo malignant transformation. Despite advancements in treatment, ovarian cancer related mortality has been relatively constant for over 30 years. Detection of ovarian cancer at later (metastasis) stages (III and IV) reduces the 5-year survival chance to 35%. Ovarian cancer often presents with non-specific symptoms, which makes it difficult to detect at early stage of disease. Therefore it is essential to obtain predictive biomarkers to help in diagnosing pre-cancerous cells. The most popular diagnostic mechanism is trans-vaginal or pelvic ultrasound followed by serum detection of cancer antigen 125 (CA125) (Scholler *et al.* 2006). CA125 is a reliable marker for monitoring the response of treatment and disease recurrence, rather than for early diagnosis. During the past decade, numerous biomarkers, independently or in conjunction with CA125, have been clinically evaluated for their cancer-associated over-expression, and their expression in normal and cancerous OSE cell lines was verified using immunohistochemical procedures. All of these biomarkers are normal cell proteins whose serum concentration can be affected by the hormonal regime that changes during different stages of the reproductive cycle. Hence, the efficacy of the biomarkers in cancer detection depends on a greater understanding of the physiological alterations in such proteins prevailing in the OSE layer, in follicular fluid and in serum depending on the paracrine and endocrine conditions regulating their expression. The work presented in this thesis has demonstrated some basic model systems that could be utilised and extended to investigate the potential of putative biomarkers. In this study, combined *in vivo* / *in vitro* methods were performed to monitor the expression of specific proliferative and cell

death makers at gene and protein level. The same line of investigation can be extended by using the above cancer-determinant biomarkers. If pregnancy and oral contraceptive drugs including the progesterone-based pill suppress tumour formation, one can judge this fact by using animal OSE cells and the cells grown in cell cultures, just by replacing the existing markers with the human cancer biomarkers. In this way one can shortlist a few valid markers that are minimally affected by the paracrine or endocrine factors, while they only respond to the conditions that lead to tumourigenesis. Among the described human biomarkers we have applied *p53* marker in the animal model and in cultured cells, and our study revealed that progesterone up-regulated the p53 protein synthesis and apoptotic destruction of those mitogenic cells that tend to undergo neoplastic growth. The same attribute in EOC patients may suppress recurrence of the disease. Thus animal models help to understand the possible hormonal therapeutic mechanism and to monitor the response of the drug using p53 biomarker. By pursuing all of these model systems, we may eventually be able to improve diagnosis and treatment.

References

Reference List

- Abbott DH, Foong SC, Barnett DK & Dumesic DA 2004 Nonhuman primates contribute unique understanding to anovulatory infertility in women. *ILAR.J.* **45** 116-131.

- Adams AT & Auersperg N 1981 Transformation of cultured rat ovarian surface epithelial cells by Kirsten murine sarcoma virus. *Cancer Res.* **41** 2063-2072.

- Adams GP, Matteri RL & Ginther OJ 1992 Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. *J.Reprod.Fertil.* **96** 627-640.

- Agca C, Ries JE, Kolath SJ, Kim JH, Forrester LJ, Antoniou E, Whitworth KM, Mathialagan N, Springer GK, Prather RS & Lucy MC 2006 Luteinization of porcine preovulatory follicles leads to systematic changes in follicular gene expression. *Reproduction.* **132** 133-145.

- Agorastos T, Vaitsi V, Paschopoulos M, Vakiani A, Zournatzi-Koiou V, Saravelos H, Kostopoulou E, Constantinidis T, Dinas K, Vavilis D, Lolis D & Bontis J 2004 Prolonged use of gonadotropin-releasing hormone agonist and tibolone as add-back therapy for the treatment of endometrial hyperplasia. *Maturitas* **48** 125-132.

- Ahmed N, Maines-Bandiera S, Quinn MA, Unger WG, Dedhar S & Auersperg N 2006 Molecular pathways regulating EGF-induced epithelio-mesenchymal transition in human ovarian surface epithelium. *Am.J.Physiol Cell Physiol* **290** C1532-C1542.

- Al Gubory KH & Abdennebi L 1996 Evidence that the conceptus contributes to the inhibition of follicular growth in the ewe. *Anim Reprod.Sci.* **45** 71-80.

- Al Gubory KH, Driancourt MA, Antoine M, Martal J & Neimer N 1994 Evidence that a non-steroidal factor from corpus luteum of pregnant sheep inhibits aromatase activity of ovarian follicles in vitro. *J.Reprod.Fertil.* **100** 51-56.

- Al-Gubory KH & Martinet J 1986 Comparison of the total ovarian follicular populations at day 140 of pregnancy and at day 5 postpartum in ewes. *Theriogenology* **25** 795-808.

- Al Gubory KH, Solari A & Mirman B 1999 Effects of luteectomy on the maintenance of pregnancy, circulating progesterone concentrations and lambing performance in sheep. *Reprod.Fertil.Dev.* **11** 317-322.

- Asdell SA 1946 *Patterns of Mammalian Reproduction.* Comstock Pub. Assoc., Ithaca.

- Auersperg N, Edelson MI, Mok SC, Johnson SW & Hamilton TC 1998 The biology of ovarian cancer. *Semin.Oncol.* **25** 281-304.

- Auersperg N, Siemens CH & Myrdal SE 1984 Human ovarian surface epithelium in primary culture. *In Vitro* **20** 743-755.

- Auersperg N, Wong AS, Choi KC, Kang SK & Leung PC 2001 Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr.Rev.* **22** 255-288.

- Auersperg N, Woo MM & Gilks CB 2008 The origin of ovarian carcinomas: a developmental view. *Gynecol.Oncol.* **110** 452-454.

- Aumailley M & Gayraud B 1998 Structure and biological activity of the extracellular matrix. *J.Mol.Med.* **76** 253-265.

- Aumailley M & Krieg T 1996 Laminins: a family of diverse multifunctional molecules of basement membranes. *J.Invest Dermatol.* **106** 209-214.

- Aunoble B, Sanches R, Didier E & Bignon YJ 2000 Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer (review). *Int.J.Oncol.* **16** 567-576.

- Babu PS, Krishnamurthy H, Chedrese PJ & Sairam MR 2000 Activation of extracellular-regulated kinase pathways in ovarian granulosa cells by the novel growth factor type 1 follicle-stimulating hormone receptor. Role in hormone signaling and cell proliferation. *J.Biol.Chem.* **275** 27615-27626.

- Baerwald AR, Adams GP & Pierson RA 2003 Characterization of ovarian follicular wave dynamics in women. *Biol.Reprod.* **69** 1023-1031.

- Baird DT & Fraser IS 1975 Concentration of oestrone and oestradiol in follicular fluid and ovarian venous blood of women. *Clin.Endocrinol.(Oxf)* **4** 259-266.

- Baker TG 1963 A Quantitative and cytological study of germ cells in human ovaries. *Proc.R.Soc.Lond B Biol.Sci.* **158** 417-433.

- Baldwin RL, Tran H & Karlan BY 1999 Primary ovarian cancer cultures are resistant to Fas-mediated apoptosis. *Gynecol.Oncol.* **74** 265-271.

- Bapat SA, Mali AM, Koppikar CB & Kurrey NK 2005 Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. *Cancer Res.* **65** 3025-3029.

- Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M & Mattioli M 2000 Vascular endothelial growth factor production in growing pig antral follicles. *Biol.Reprod.* **63** 858-864.

- Barrera JE, Shroyer KR, Said S, Hoernig G, Melrose R, Freedman PD, Wright TA, Greer RO 2008 Estrogen and Progesterone Receptor and p53 Gene Expression in Adenoid Cystic Cancer. *Head and Neck Pathol.* **2** 13–18.

- Bartlewski PM, Beard AP, Cook SJ, Chandolia RK, Honaramooz A & Rawlings NC 1999 Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. *J.Reprod.Fertil.* **115** 111-124.

- Bellin ME, Hinshelwood MM, Hauser ER & Ax RL 1984 Influence of suckling and side of corpus luteum or pregnancy on folliculogenesis in postpartum cows. *Biol.Reprod.* **31** 849-855.

- Beral V, Bull D, Green J & Reeves G 2007 Ovarian cancer and hormone replacement therapy in the Million Women Study. *Lancet* **369** 1703-1710.

- Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, Clarke-Pearson DL & Bast RC, Jr. 1992 Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-beta. *Am.J.Obstet.Gynecol.* **166** 676-684.

- Berchuck A, Kohler MF, Marks JR, Wiseman R, Boyd J & Bast RC, Jr. 1994 The p53 tumor suppressor gene frequently is altered in gynecologic cancers. *Am.J.Obstet.Gynecol.* **170** 246-252.

- Berchuck A, Rodriguez GC, Kamel A, Dodge RK, Soper JT, Clarke-Pearson DL & Bast RC, Jr. 1991 Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer. I. Correlation of receptor expression with prognostic factors in patients with ovarian cancer. *Am.J.Obstet.Gynecol.* **164** 669-674.

- Bergeron L, Perez GI, Macdonald G, Shi L, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JC, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL & Yuan J 1998 Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes Dev.* **12** 1304-1314.

- Bister JL & Paquay R 1983 Fluctuations in the plasma levels of the follicle-stimulating hormone during estrous cycle, anestrus, gestation and lactation in the ewe: Evidence for an endogenous rhythm of FSH release. *Theriogenology* **19** 565-582.

- Bjersing L & Cajander S 1975 Ovulation and the role of the ovarian surface epithelium. *Experientia* **31** 605-608.

- Boone DL & Tsang BK 1998 Caspase-3 in the rat ovary: localization and possible role in follicular atresia and luteal regression. *Biol.Reprod.* **58** 1533-1539.

- Braithwaite AW, Sturzbecher HW, Addison C, Palmer C, Rudge K & Jenkins JR 1987 Mouse p53 inhibits SV40 origin-dependent DNA replication. *Nature* **329** 458-460.

- Bruno JB, Celestino JJ, Lima-Verde IB, Lima LF, Matos MH, Araujo VR, Saraiva MV, Martins FS, Name KP, Campello CC, Bao SN, Silva JR & Figueiredo JR 2009 Expression of vascular endothelial growth factor (VEGF) receptor in goat ovaries and improvement of in vitro caprine preantral follicle survival and growth with VEGF. *Reprod.Fertil.Dev.* **21** 679-687.

- Bu SZ, Yin DL, Ren XH, Jiang LZ, Wu ZJ, Gao QR & Pei G 1997 Progesterone induces apoptosis and up-regulation of p53 expression in human ovarian carcinoma cell lines. *Cancer* **79** 1944-1950.

- Burdette JE, Kurley SJ, Kilen SM, Mayo KE & Woodruff TK 2006 Gonadotropin-induced superovulation drives ovarian surface epithelia proliferation in CD1 mice. *Endocrinology* **147** 2338-2345.

- Burdette JE, Oliver RM, Ulyanov V, Kilen SM, Mayo KE & Woodruff TK 2007 Ovarian epithelial inclusion cysts in chronically superovulated CD1 and Smad2 dominant-negative mice. *Endocrinology* **148** 3595-3604.

- Byskov AG, Faddy MJ, Lemmen JG & Andersen CY 2005 Eggs forever? *Differentiation* **73** 438-446.

- Byskov AG, Skakkebaek NE, Stafanger G & Peters H 1977 Influence of ovarian surface epithelium and rete ovarii on follicle formation. *J.Anat.* **123** 77-86.

- Campbell BK, Souza CJ, Skinner AJ, Webb R & Baird DT 2006 Enhanced response of granulosa and theca cells from sheep carriers of the FecB mutation in vitro to gonadotropins and bone morphogenic protein-2, -4, and -6. *Endocrinology* **147** 1608-1620.

- Campbell BK 2009 The endocrine and local control of ovarian follicle development in the ewe. *Anim. Reprod.* **6 n.1**159-171.

- Campbell BK, Kendall NR & Baird DT 2009 Effect of direct ovarian infusion of bone morphogenetic protein 6 (BMP6) on ovarian function in sheep. *Biol.Reprod.* **81** 1016-1023.

- Carambula SF, Matikainen T, Lynch MP, Flavell RA, Goncalves PB, Tilly JL & Rueda BR 2002 Caspase-3 is a pivotal mediator of apoptosis during regression of the ovarian corpus luteum. *Endocrinology* **143** 1495-1501.

- Carcangiu ML & Chambers JT 1992 Sex steroid receptors in gynecologic neoplasms. *Pathol.Annu.* **27 Pt 2** 121-151.

- Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen AP & Hovatta O 2006 Anti-Mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. *Hum.Reprod.* **21** 2223-2227.

- Chaffin CL, Hess DL & Stouffer RL 1999 Dynamics of periovulatory steroidogenesis in the rhesus monkey follicle after ovarian stimulation. *Hum.Reprod.* **14** 642-649.

- Chamoun D, Choi D, Tavares AB, Udoff LC, Levitas E, Resnick CE, Rosenfeld RG & Adashi EY 2002 Regulation of granulosa cell-derived insulin-like growth factor binding proteins (IGFBPs): role for protein kinase-C in the pre- and posttranslational modulation of IGFBP-4 and IGFBP-5. *Biol.Reprod.* **67** 1003-1012.

- Cheng KW, Lu Y & Mills GB 2005 Assay of Rab25 function in ovarian and breast cancers. *Methods Enzymol.* **403** 202-215.

- Choi JH, Wong AS, Huang HF & Leung PC 2007 Gonadotropins and ovarian cancer. *Endocr.Rev.* **28** 440-461.

- Clement PB 1987 Histology of the ovary. *Am.J.Surg.Pathol.* **11** 277-303.

- Clow OL, Hurst PR & Fleming JS 2002 Changes in the mouse ovarian surface epithelium with age and ovulation number. *Mol.Cell Endocrinol.* **191** 105-111.

- Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC & Hamilton TC 2003 Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. *Cancer Res.* **63** 1389-1397.

- Couchman JR & Woods A 1993 Structure and biology of pericellular proteoglycans. In Roberts DD, Mecham RR, eds. Cell Surface and Extracellular Glycoconjugates. San Diego, Academic Press, 33-82

- Corney DC, Flesken-Nikitin A, Godwin AK, Wang W & Nikitin AY 2007 MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer Res.* **67** 8433-8438.

- Cramer DW & Welch WR 1983 Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J.Natl.Cancer Inst.* **71** 717-721.

- Davidson TR, Chamberlain CS, Bridges TS & Spicer LJ 2002 Effect of follicle size on in vitro production of steroids and insulin-like growth factor (IGF)-I, IGF-II, and the IGF-binding proteins by equine ovarian granulosa cells. *Biol.Reprod.* **66** 1640-1648.

- Davies BR, Worsley SD & Ponder BA 1998 Expression of E-cadherin, alpha-catenin and beta-catenin in normal ovarian surface epithelium and epithelial ovarian cancers. *Histopathology* **32** 69-80.

- De los RM, Villagran ML, Cepeda R, Duchens M, Parraguez V & Urquieta B 2006 Histological characteristics and steroid concentration of ovarian follicles at different stages of development in pregnant and non-pregnant dairy cows. *Vet.Res.Commun.* **30** 161-173.

- Deghenghi R, Boutignon F, Wuthrich P & Lenaerts V 1993 Antarelix (EP 24332) a novel water soluble LHRH antagonist. *Biomed.Pharmacother.* **47** 107-110.

- Deligdisch L, Einstein AJ, Guera D & Gil J 1995 Ovarian dysplasia in epithelial inclusion cysts. A morphometric approach using neural networks. *Cancer* **76** 1027-1034.

- D'Haeseleer M, Cornillie P, Simoens P & van den BW 2006 Localization of oestrogen receptors within various bovine ovarian cell types at different stages of the oestrous cycle. *Anat.Histol.Embryol.* **35** 334-342.

- D'Haeseleer M, Simoens P & van den BW 2007 Cell-specific localization of progesterone receptors in the bovine ovary at different stages of the oestrous cycle. *Anim Reprod.Sci.* **98** 271-281.

- Diaz FJ, Wigglesworth K & Eppig JJ 2007 Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice. *Dev.Biol.* **305** 300-311.

- Dickson SE & Fraser HM 2000 Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate. *J.Clin.Endocrinol.Metab* **85** 2339-2344.

- Dieleman SJ, Kruip TA, Fontijne P, de Jong WH & van der Weyden GC 1983 Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. *J.Endocrinol.* **97** 31-42.

- Doraiswamy V, Parrott JA & Skinner MK 2000 Expression and action of transforming growth factor alpha in normal ovarian surface epithelium and ovarian cancer. *Biol.Reprod.* **63** 789-796.

- Drapkin R & Hecht JL 2002 The origins of ovarian cancer: Hurdles and progress. *Women's Oncol Rev* **2** 261-268.

- Drapkin R & Hecht JL 2006 "Pathogenesis of ovarian cancer," in *Diagnostic gynecologic and obstetric pathology*, Elsevier Saunders, Philadelphia, Pa, USA.

- Dubeau L 1999 The cell of origin of ovarian epithelial tumors and the ovarian surface epithelium dogma: does the emperor have no clothes? *Gynecol.Oncol.* **72** 437-442.

- Dubeau L 2008 BRCA1-induced ovarian oncogenesis. *Adv.Exp.Med.Biol.* **622** 89-97.

- Duncan MB & Kalluri R 2009 Parstatin, a novel protease-activated receptor 1-derived inhibitor of angiogenesis. *Mol.Interv.* **9** 168-170.

- Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA & Themmen AP 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology* **142** 4891-4899.

- Durlinger AL, Visser JA & Themmen AP 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction.* **124** 601-609.

- Eggan K, Jurga S, Gosden R, Min IM & Wagers AJ 2006 Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature* **441** 1109-1114.

- Elmore S 2007 Apoptosis: a review of programmed cell death. *Toxicol.Pathol.* **35** 495-516.

- Emons G, Grundker C, Gunthert AR, Westphalen S, Kavanagh J & Verschraegen C 2003 GnRH antagonists in the treatment of gynecological and breast cancers. *Endocr.Relat Cancer* **10** 291-299.

- Epifano O, Liang LF, Familari M, Moos MC, Jr. & Dean J 1995 Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* **121** 1947-1956.

- Eppig JJ 1991 Intercommunication between mammalian oocytes and companion somatic cells. *Bioessays* **13** 569-574.

- Erickson GF & Shimasaki S 2000 The role of the oocyte in folliculogenesis. *Trends Endocrinol.Metab* **11** 193-198.

- Faddy MJ 2000 Follicle dynamics during ovarian ageing. *Mol.Cell Endocrinol.* **163** 43-48.

- Faddy MJ & Gosden RG 1996 A model conforming the decline in follicle numbers to the age of menopause in women. *Hum.Reprod.* **11** 1484-1486.

- Faddy MJ, Gosden RG, Gougeon A, Richardson SJ & Nelson JF 1992 Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Hum.Reprod.* **7** 1342-1346.

- Fathalla MF 1971 Incessant ovulation--a factor in ovarian neoplasia? *Lancet* **2** 163.

- Feeley KM & Wells M 2001 Precursor lesions of ovarian epithelial malignancy. *Histopathology* **38** 87-95.

- Ferrara N, Frantz G, LeCouter J, Dillard-Telm L, Pham T, Draksharapu A, Giordano T & Peale F 2003 Differential expression of the angiogenic factor genes vascular endothelial growth factor (VEGF) and endocrine gland-derived VEGF in normal and polycystic human ovaries. *Am.J.Pathol.* **162** 1881-1893.

- Fister S, Gunthert AR, Emons G & Grundker C 2007 Gonadotropin-releasing hormone type II antagonists induce apoptotic cell death in human endometrial and ovarian cancer cells in vitro and in vivo. *Cancer Res.* **67** 1750-1756.

- Fleming JS, Beaugie CR, Haviv I, Chenevix-Trench G & Tan OL 2006 Incessant ovulation, inflammation and epithelial ovarian carcinogenesis: revisiting old hypotheses. *Mol.Cell Endocrinol.* **247** 4-21.

- Fortune JE & Vincent SE 1983 Progesterone inhibits the induction of aromatase activity in rat granulosa cells in vitro. *Biol.Reprod.* **28** 1078-1089.

- Fraser HM & Duncan WC 2009 SRB Reproduction, Fertility and Development Award Lecture 2008. Regulation and manipulation of angiogenesis in the ovary and endometrium. *Reprod.Fertil.Dev.* **21** 377-392.

- Fraser HM, Wilson H, Wulff C, Rudge JS & Wiegand SJ 2006 Administration of vascular endothelial growth factor Trap during the 'post-angiogenic' period of the luteal phase causes rapid functional luteolysis and selective endothelial cell death in the marmoset. *Reproduction.* **132** 589-600.

- Fredrickson TN 1987 Ovarian tumors of the hen. *Environ.Health Perspect.* **73** 35-51.

- Fukuda M, Katayama K & Tojo S 1980 Inhibitory effect of progesterone on follicular growth and induced superovulation in the rat. *Arch.Gynecol.* **230** 77-87.

- Gaytan M, Sanchez MA, Morales C, Bellido C, Millan Y, Martin de Las MJ, Sanchez-Criado JE & Gaytan F 2005 Cyclic changes of the ovarian surface epithelium in the rat. *Reproduction.* **129** 311-321.

- Ghahremani M, Foghi A & Dorrington JH 1999 Etiology of ovarian cancer: a proposed mechanism. *Med.Hypotheses* **52** 23-26.

- Gilchrist RB, Wicherek M, Heistermann M, Nayudu PL & Hodges JK 2001 Changes in follicle-stimulating hormone and follicle populations during the ovarian cycle of the common marmoset. *Biol.Reprod.* **64** 127-135.

- Gillett WR, Mitchell A & Hurst PR 1991 A scanning electron microscopic study of the human ovarian surface epithelium: characterization of two cell types. *Hum.Reprod.* **6** 645-650.

- Ginther OJ 1993 Major and minor follicular waves during the equine estrous cycle. *J Equine Vet Sci*. **13** 18–25.

- Ginther OJ, Kot K, Kulick LJ, Martin S & Wiltbank MC 1996 Relationships between FSH and ovarian follicular waves during the last six months of pregnancy in cattle. *J.Reprod.Fertil.* **108** 271-279.

- Godwin AK, Testa JR & Hamilton TC 1993 The biology of ovarian cancer development. *Cancer* **71** 530-536.

- Gook DA, Edgar DH, Borg J & Martic M 2008 Detection of zona pellucida proteins during human folliculogenesis. *Hum.Reprod.* **23** 394-402.

- Gosden RG 2002 Oogenesis as a foundation for embryogenesis. *Mol.Cell Endocrinol.* **186** 149-153.

- Gosden RG 2004 Germline stem cells in the postnatal ovary: is the ovary more like a testis? *Hum.Reprod.Update.* **10** 193-195.

- Gotfredson GS & Murdoch WJ 2007 Morphologic responses of the mouse ovarian surface epithelium to ovulation and steroid hormonal milieu. *Exp.Biol.Med.(Maywood.)* **232** 277-280.

- Gougeon A 1996 Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr.Rev.* **17** 121-155.

- Greenwald GS, Keever JE & Grady KL 1967 Ovarian morphology and pituitary FSH and LH concentration in the pregnant and lactating hamster. *Endocrinology* **80** 851-856.

- Gubbay O, Guo W, Rae MT, Niven D, Howie AF, McNeilly AS, Xu L, Hillier SG 2004 Anti-inflammatory and proliferative responses in human and ovine ovarian surface epithelial cells. *Reproduction.* **128** 607-614.

- Gubbay O, Rae MT, McNeilly AS, Donadeu FX, Zeleznik AJ & Hillier SG 2006 cAMP response element-binding (CREB) signalling and ovarian surface epithelial cell survival. *J.Endocrinol.* **191** 275-285.

- Guilbault LA, Dufour JJ, Thatcher WW, Drost M & Haibel GK 1986 Ovarian follicular development during early pregnancy in cattle. *J.Reprod.Fertil.* **78** 127-135.

- Hafez ES, Makabe S & Motta PM 1980 Surface ultrastructure of functional and nonfunctional human ovaries. *Int.J.Fertil.* **25** 94-99.

- Hafez ESE 1952 Studies on the breeding season and reproduction of the ewe. *J Ag Sci* **42** 189-265.

- Hankinson SE, Colditz GA, Hunter DJ, Spencer TL, Rosner B & Stampfer MJ 1992 A quantitative assessment of oral contraceptive use and risk of ovarian cancer. *Obstet.Gynecol.* **80** 708-714.

- Harlow CR, Hearn JP & Hodges JK 1984 Ovulation in the marmoset monkey: endocrinology, prediction and detection. *J.Endocrinol.* **103** 17-24.

- Hay ED 1995 An overview of epithelio-mesenchymal transformation. *Acta Anat.(Basel)* **154** 8-20.

- Hayden C 2008 GnRH analogues: applications in assisted reproductive techniques. *Eur.J.Endocrinol.* **159 Suppl 1** S17-S25.

- Henricks DM, Dickey JF & Niswender GD 1970 Serum luteinizing hormone and plasma progesterone levels during the estrous cycle and early pregnancy in cows. *Biol.Reprod.* **2** 346-351.

- Herbst KL 2003 Gonadotropin-releasing hormone antagonists. *Curr.Opin.Pharmacol.* **3** 660-666.

- Hillier SG 1985 Sex steroid metabolism and follicular development in the ovary. *Oxf Rev.Reprod.Biol.* **7** 168-222.

- Hillier SG 2001 Gonadotropic control of ovarian follicular growth and development. *Mol.Cell Endocrinol.* **179** 39-46.

- Hillier SG, Anderson RA, Williams AR & Tetsuka M 1998 Expression of oestrogen receptor alpha and beta in cultured human ovarian surface epithelial cells. *Mol.Hum.Reprod.* **4** 811-815.

- Hirakawa T, Minegishi T, Abe K, Kishi H, Ibuki Y & Miyamoto K 1999 A role of insulin-like growth factor I in luteinizing hormone receptor expression in granulosa cells. *Endocrinology* **140** 4965-4971.

- Hirshfield AN 1991 Development of follicles in the mammalian ovary. *Int.Rev.Cytol.* **124** 43-101.

- Howles CM 2000 Role of LH and FSH in ovarian function. *Mol.Cell Endocrinol.* **161** 25-30.

- Hu Z & Deng X 2000 [The effect of progesterone on proliferation and apoptosis in ovarian cancer cell]. *Zhonghua Fu Chan Ke.Za Zhi.* **35** 423-426.

- Hudson LG, Zeineldin R & Stack MS 2008 Phenotypic plasticity of neoplastic ovarian epithelium: unique cadherin profiles in tumor progression. *Clin.Exp.Metastasis* **25** 643-655.

- Huirne JA, van Loenen AC, Schats R, McDonnell J, Hompes PG, Schoemaker J, Homburg R & Lambalk CB 2004 Dose-finding study of daily gonadotropin-releasing hormone (GnRH) antagonist for the prevention of premature luteinizing hormone surges in IVF/ICSI patients: antide and hormone levels. *Hum.Reprod.* **19** 2206-2215.

- Hulshof SC, Figueiredo JR, Beckers JF, Bevers MM & van den HR 1994 Isolation and characterization of preantral follicles from foetal bovine ovaries. *Vet.Q.* **16** 78-80.

- Hulshof SC, Figueiredo JR, Beckers JF, Bevers MM, van der Donk JA & van den HR 1995 Effects of fetal bovine serum, FSH and 17beta-estradiol on the culture of bovine preantral follicles. *Theriogenology* **44** 217-226.

- Hussein TS, Froiland DA, Amato F, Thompson JG & Gilchrist RB 2005 Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *J.Cell Sci.* **118** 5257-5268.

- Hutt KJ & Albertini DF 2007 An oocentric view of folliculogenesis and embryogenesis. *Reprod.Biomed.Online.* **14** 758-764.

- Hutt KJ, McLaughlin EA & Holland MK 2006 Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. *Mol.Hum.Reprod.* **12** 61-69.

- Ivarsson K, Sundfeldt K, Brannstrom M & Janson PO 2001 Production of steroids by human ovarian surface epithelial cells in culture: possible role of progesterone as growth inhibitor. *Gynecol.Oncol.* **82** 116-121.

- Jackson KS, Inoue K, Davis DA, Hilliard TS & Burdette JE 2009 Three-dimensional ovarian organ culture as a tool to study normal ovarian surface epithelial wound repair. *Endocrinology* **150** 3921-3926.

- Jacobs I & Bast RC, Jr. 1989 The CA 125 tumour-associated antigen: a review of the literature. *Hum.Reprod.* **4** 1-12.

- Jensen A, Sharif H, Frederiksen K & Kjaer SK 2009 Use of fertility drugs and risk of ovarian cancer: Danish Population Based Cohort Study. *BMJ* **338** b249.

- Johnson AL & Bridgham JT 2000 Caspase-3 and -6 expression and enzyme activity in hen granulosa cells. *Biol.Reprod.* **62** 589-598.

- Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT & Tilly JL 2005 Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* **122** 303-315.

- Johnson J, Canning J, Kaneko T, Pru JK & Tilly JL 2004 Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* **428** 145-150.

- Kabawat SE, Bast RC, Jr., Bhan AK, Welch WR, Knapp RC & Colvin RB 1983 Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125. *Int.J.Gynecol.Pathol.* **2** 275-285.

- Kang SK, Choi KC, Cheng KW, Nathwani PS, Auersperg N & Leung PC 2000 Role of gonadotropin-releasing hormone as an autocrine growth factor in human ovarian surface epithelium. *Endocrinology* **141** 72-80.

- Karlan BY, Jones J, Greenwald M & Lagasse LD 1995 Steroid hormone effects on the proliferation of human ovarian surface epithelium in vitro. *Am.J.Obstet.Gynecol.* **173** 97-104.

- Katabuchi H & Okamura H 2003 Cell biology of human ovarian surface epithelial cells and ovarian carcinogenesis. *Med.Electron Microsc.* **36** 74-86.

- Kenny HA, Kaur S, Coussens LM & Lengyel E 2008 The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin. *J.Clin.Invest* **118** 1367-1379.

- Key TJ 1995 Hormones and cancer in humans. *Mutat.Res.* **333** 59-67.

- Kisliouk T, Podlovni H, Spanel-Borowski K, Ovadia O, Zhou QY & Meidan R 2005 Prokineticins (endocrine gland-derived vascular endothelial growth factor and BV8) in the bovine ovary: expression and role as mitogens and survival factors for corpus luteum-derived endothelial cells. *Endocrinology* **146** 3950-3958.

- Knecht M, Darbon JM, Ranta T, Baukal AJ & Catt KJ 1984 Estrogens enhance the adenosine 3',5'-monophosphate-mediated induction of follicle-stimulating hormone and luteinizing hormone receptors in rat granulosa cells. *Endocrinology* **115** 41-49.

- Krammer PH 1999 CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv.Immunol.* **71** 163-210.

- Kruk PA, Uitto VJ, Firth JD, Dedhar S & Auersperg N 1994 Reciprocal interactions between human ovarian surface epithelial cells and adjacent extracellular matrix. *Exp.Cell Res.* **215** 97-108.

- Kuerbitz SJ, Plunkett BS, Walsh WV & Kastan MB 1992 Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc.Natl.Acad.Sci.U.S.A* **89** 7491-7495.

- Kundranda MN, Henderson M, Carter KJ, Gorden L, Binhazim A, Ray S, Baptiste T, Shokrani M, Leite-Browning ML, Jahnen-Dechent W, Matrisian LM & Ochieng J 2005 The serum glycoprotein fetuin-A promotes Lewis lung carcinoma tumorigenesis via adhesive-dependent and adhesive-independent mechanisms. *Cancer Res.* **65** 499-506.

- Landen CN, Jr., Birrer MJ & Sood AK 2008 Early events in the pathogenesis of epithelial ovarian cancer. *J.Clin.Oncol.* **26** 995-1005.

- Lane DP 1992 Cancer. p53, guardian of the genome. *Nature* **358** 15-16.

- Lee JM, Dedhar S, Kalluri R & Thompson EW 2006 The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J.Cell Biol.* **172** 973-981.

- Lee CJ, Ariztia EV & Fishman DA 2007 Conventional and proteomic technologies for the detection of early stage malignancies: markers for ovarian cancer. *Crit Rev.Clin.Lab Sci.* **44** 87-114.

- Lenton EA, King H, Thomas EJ, Smith SK, McLachlan RI, MacNeil S & Cooke ID 1988 The endocrine environment of the human oocyte. *J.Reprod.Fertil.* **82** 827-841.

- Leung PC & Choi JH 2007 Endocrine signaling in ovarian surface epithelium and cancer. *Hum.Reprod.Update.* **13** 143-162.

- Levanon K, Crum C & Drapkin R 2008 New insights into the pathogenesis of serous ovarian cancer and its clinical impact. *J.Clin.Oncol.* **26** 5284-5293.

- Levine AJ 1997 p53, the cellular gatekeeper for growth and division. *Cell* **88** 323-331.

- Li R, Phillips DM & Mather JP 1995 Activin promotes ovarian follicle development in vitro. *Endocrinology* **136** 849-856.

- Li Q, McKenzie LJ & Matzuk MM 2008 Revisiting oocyte-somatic cell interactions: in search of novel intrafollicular predictors and regulators of oocyte developmental competence. *Mol.Hum.Reprod.* **14** 673-678.

- Lind AK, Weijdegard B, Dahm-Kahler P, Molne J, Sundfeldt K & Brannstrom M 2006 Collagens in the human ovary and their changes in the perifollicular stroma during ovulation. *Acta Obstet.Gynecol.Scand.* **85** 1476-1484.

- Linzell JL & Heap RB 1968 A comparison of progesterone metabolism in the pregnant sheep and goat: sources of production and an estimation of uptake by some target organs. *J.Endocrinol.* **41** 433-438.

- Livneh E & Fishman DD 1997 Linking protein kinase C to cell-cycle control. *Eur.J.Biochem.* **248** 1-9.

- Lukanova A, Lundin E, Akhmedkhanov A, Micheli A, Rinaldi S, Zeleniuch-Jacquotte A, Lenner P, Muti P, Biessy C, Krogh V, Berrino F, Hallmans G, Riboli E, Kaaks R & Toniolo P 2003 Circulating levels of sex steroid hormones and risk of ovarian cancer. *Int.J.Cancer* **104** 636-642.

- MacCalman CD, Farookhi R & Blaschuk OW 1994 Estradiol regulates E-cadherin mRNA levels in the surface epithelium of the mouse ovary. *Clin.Exp.Metastasis* **12** 276-282.

- Maines-Bandiera SL & Auersperg N 1997 Increased E-cadherin expression in ovarian surface epithelium: an early step in metaplasia and dysplasia? *Int.J.Gynecol.Pathol.* **16** 250-255.

- Makrigiannakis A, Coukos G, Christofidou-Solomidou M, Gour BJ, Radice GL, Blaschuk O & Coutifaris C 1999 N-cadherin-mediated human granulosa cell

adhesion prevents apoptosis: a role in follicular atresia and luteolysis? *Am.J.Pathol.* **154** 1391-1406.

- Mamputha S, Lu ZL, Roeske RW, Millar RP, Katz AA & Flanagan CA 2007 Conserved amino acid residues that are important for ligand binding in the type I gonadotropin-releasing hormone (GnRH) receptor are required for high potency of GnRH II at the type II GnRH receptor. *Mol.Endocrinol.* **21** 281-292.

- Marchant J 1980 Animal models for tumors of the ovary. In: Murphy ED, Beamer WG (Eds.), Biology of Ovarian Neoplasia. **50** 50–65.

- Marion P, Lapeyre D, de Bennetot M, Cottin M, Estanove S, George M, Rubet A & Pinet F 1971 [Regulation of a portacaval anastomosis with an implanted valve animated by a magnetic source]. *Lyon.Chir* **67** 383-385.

- Masters JR 2000 *Animal Cell Culture. A practical Approach.* 3rd Ed., Oxford University Press.

- Matikainen T, Perez GI, Zheng TS, Kluzak TR, Rueda BR, Flavell RA & Tilly JL 2001 Caspase-3 gene knockout defines cell lineage specificity for programmed cell death signaling in the ovary. *Endocrinology* **142** 2468-2480.

- McCormick D, Chong H, Hobbs C, Datta C & Hall PA 1993 Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. *Histopathology* **22** 355-360.

- McKeehan WL, Adams PS & Rosser MP 1984 Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. *Cancer Res.* **44** 1998-2010.

- McNatty KP, Heath DA, Lundy T, Fidler AE, Quirke L, O'Connell A, Smith P, Groome N & Tisdall DJ 1999 Control of early ovarian follicular development. *J.Reprod.Fertil.Suppl* **54** 3-16.

- McNeil L, Hobson S, Nipper V & Rodland KD 1998 Functional calcium-sensing receptor expression in ovarian surface epithelial cells. *Am.J.Obstet.Gynecol.* **178** 305-313.

- Meisler JG 2000 Toward optimal health: the experts discuss ovarian cancer. *J.Womens Health Gend.Based.Med.* **9** 705-710.

- Metallinou C, Asimakopoulos B, Schroer A & Nikolettos N 2007 Gonadotropin-releasing hormone in the ovary. *Reprod.Sci.* **14** 737-749.

- Millar R, Lowe S, Conklin D, Pawson A, Maudsley S, Troskie B, Ott T, Millar M, Lincoln G, Sellar R, Faurholm B, Scobie G, Kuestner R, Terasawa E & Katz A 2001 A novel mammalian receptor for the evolutionarily conserved type II GnRH. *Proc.Natl.Acad.Sci.U.S.A* **98** 9636-9641.

- Millar RP 2005 GnRHs and GnRH receptors. *Anim Reprod.Sci.* **88** 5-28.

- Moll R 1998 Cytokeratins as markers of differentiation in the diagnosis of epithelial tumors. *Subcell.Biochem.* **31** 205-262.

- Murdoch WJ 1994 Ovarian surface epithelium during ovulatory and anovulatory ovine estrous cycles. *Anat.Rec.* **240** 322-326.

- Murdoch WJ 1996 Ovarian surface epithelium, ovulation and carcinogenesis. *Biol.Rev.Camb.Philos.Soc.* **71** 529-543.

- Murdoch WJ 2005 Carcinogenic potential of ovulatory genotoxicity. *Biol.Reprod.* **73** 586-590.

- Murdoch J, Van Kirk EA & Murdoch WJ 1999 Hormonal control of urokinase plasminogen activator secretion by sheep ovarian surface epithelial cells. *Biol.Reprod.* **61** 1487-1491.

- Murdoch WJ & McDonnel AC 2002 Roles of the ovarian surface epithelium in ovulation and carcinogenesis. *Reproduction.* **123** 743-750.

- Murdoch WJ & Van Kirk EA 2001 Estrogenic upregulation of DNA polymerase beta in oocytes of preovulatory ovine follicles. *Mol.Reprod.Dev.* **58** 417-423.

- Murdoch WJ, Van Kirk EA & Alexander BM 2005 DNA damages in ovarian surface epithelial cells of ovulatory hens. *Exp.Biol.Med.(Maywood.)* **230** 429-433.

- Murdoch WJ, Wilken C & Young DA 1999 Sequence of apoptosis and inflammatory necrosis within the formative ovulatory site of sheep follicles. *J.Reprod.Fertil.* **117** 325-329.

- Muskhelishvili L, Wingard SK & Latendresse JR 2005 Proliferating cell nuclear antigen--a marker for ovarian follicle counts. *Toxicol.Pathol.* **33** 365-368.

- Negri E, Franceschi S, Tzonou A, Booth M, La Vecchia C, Parazzini F, Beral V, Boyle P & Trichopoulos D 1991 Pooled analysis of 3 European case-control studies: I. Reproductive factors and risk of epithelial ovarian cancer. *Int.J.Cancer* **49** 50-56.

- Ness RB, Grisso JA, Klapper J, Schlesselman JJ, Silberzweig S, Vergona R, Morgan M & Wheeler JE 2000 Risk of ovarian cancer in relation to estrogen and

progestin dose and use characteristics of oral contraceptives. SHARE Study Group. Steroid Hormones and Reproductions. *Am.J.Epidemiol.* **152** 233-241.

- Ness RB, Cramer DW, Goodman MT, Kjaer SK, Mallin K, Mosgaard BJ, Purdie DM, Risch HA, Vergona R & Wu AH 2002 Infertility, fertility drugs, and ovarian cancer: a pooled analysis of case-control studies. *Am.J.Epidemiol.* **155** 217-224.

- Newton H, Picton H & Gosden RG 1999 In vitro growth of oocyte-granulosa cell complexes isolated from cryopreserved ovine tissue. *J.Reprod.Fertil.* **115** 141-150.

- Nichols AF & Sancar A 1992 Purification of PCNA as a nucleotide excision repair protein. *Nucleic Acids Res.* **20** 2441-2446.

- Nicosia SV, Narconis RJ & Saunders BO 1989 Regulation and temporal sequence of surface epithelium morphogenesis in the postovulatory rabbit ovary. *Prog.Clin.Biol.Res.* **296** 111-119.

- Nilsson E, Doraiswamy V, Parrott JA & Skinner MK 2001a Expression and action of transforming growth factor beta (TGFbeta1, TGFbeta2, TGFbeta3) in normal bovine ovarian surface epithelium and implications for human ovarian cancer. *Mol.Cell Endocrinol.* **182** 145-155.

- Nilsson E, Parrott JA & Skinner MK 2001b Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol.Cell Endocrinol.* **175** 123-130.

- Nilsson E, Rogers N & Skinner MK 2007 Actions of anti-Mullerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition. *Reproduction.* **134** 209-221.

- Nilsson EE, Detzel C & Skinner MK 2006 Platelet-derived growth factor modulates the primordial to primary follicle transition. *Reproduction.* **131** 1007-1015.

- Ohshima K, Ohshima K, Arai KY, Kishi H, Itoh M, Watanabe G, Terranova PF, Arai K, Uehara K, Groome NP & Taya K 2002 Potential role of activin A in follicular development during the second half of pregnancy in the golden hamster: utero-placental source of activin A. *J.Endocrinol.* **172** 247-253.

- Osterholzer HO, Johnson JH & Nicosia SV 1985 An autoradiographic study of rabbit ovarian surface epithelium before and after ovulation. *Biol.Reprod.* **33** 729-738.

- Otsuka F, Moore RK & Shimasaki S 2001 Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. *J.Biol.Chem.* **276** 32889-32895.

- Pan Y & Huang X 2008 Epithelial ovarian cancer stem cells-a review. *Int.J.Clin.Exp.Med.* **1** 260-266.

- Parrott JA & Skinner MK 1997 Direct actions of kit-ligand on theca cell growth and differentiation during follicle development. *Endocrinology* **138** 3819-3827.

- Parrott JA & Skinner MK 1998 Thecal cell-granulosa cell interactions involve a positive feedback loop among keratinocyte growth factor, hepatocyte growth factor, and Kit ligand during ovarian follicular development. *Endocrinology* **139** 2240-2245.

- Parrott JA & Skinner MK 2000 Expression and action of hepatocyte growth factor in human and bovine normal ovarian surface epithelium and ovarian cancer. *Biol.Reprod.* **62** 491-500.

- Parrott JA, Doraiswamy V, Kim G, Mosher R & Skinner MK 2001 Expression and actions of both the follicle stimulating hormone receptor and the luteinizing hormone receptor in normal ovarian surface epithelium and ovarian cancer. *Mol.Cell Endocrinol.* **172** 213-222.

- Parrott JA, Kim G & Skinner MK 2000a Expression and action of kit ligand/stem cell factor in normal human and bovine ovarian surface epithelium and ovarian cancer. *Biol.Reprod.* **62** 1600-1609.

- Parrott JA, Mosher R, Kim G & Skinner MK 2000b Autocrine interactions of keratinocyte growth factor, hepatocyte growth factor, and kit-ligand in the regulation of normal ovarian surface epithelial cells. *Endocrinology* **141** 2532-2539.

- Peralta SA, Knudsen KA, Jaurand MC, Johnson KR, Wheelock MJ, Klein-Szanto AJ & Salazar H 1995 The differential expression of N-cadherin and E-cadherin distinguishes pleural mesotheliomas from lung adenocarcinomas. *Hum.Pathol.* **26** 1363-1369.

- Pon YL, Auersperg N & Wong AS 2005 Gonadotropins regulate N-cadherin-mediated human ovarian surface epithelial cell survival at both post-translational and transcriptional levels through a cyclic AMP/protein kinase A pathway. *J.Biol.Chem.* **280** 15438-15448.

- Ponnusamy MP & Batra SK 2008 Ovarian cancer: emerging concept on cancer stem cells. *J.Ovarian.Res.* **1** 4.

- Quirk SM, Cowan RG & Huber SH 1997 Fas antigen-mediated apoptosis of ovarian surface epithelial cells. *Endocrinology* **138** 4558-4566.

- Rae MT, Niven D, Critchley HO, Harlow CR & Hillier SG 2004 Antiinflammatory steroid action in human ovarian surface epithelial cells. *J.Clin.Endocrinol.Metab* **89** 4538-4544.

- Rae MT, Price D, Harlow CR, Critchley HO & Hillier SG 2009 Glucocorticoid receptor-mediated regulation of MMP9 gene expression in human ovarian surface epithelial cells. *Fertil.Steril.* **92** 703-708.

- Rao BR & Slotman BJ 1991 Endocrine factors in common epithelial ovarian cancer. *Endocr.Rev.* **12** 14-26.

- Redmer DA, Doraiswamy V, Bortnem BJ, Fisher K, Jablonka-Shariff A, Grazul-Bilska AT & Reynolds LP 2001 Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. *Biol.Reprod.* **65** 879-889.

- Reynolds LP, Killilea SD & Redmer DA 1992 Angiogenesis in the female reproductive system. *FASEB J.* **6** 886-892.

- Risch HA 1998 Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *J.Natl.Cancer Inst.* **90** 1774-1786.

- Rizzo A, Spedicato M, Minoia G, Mutinati M, Cinone M, Jirillo F & Sciorsci RL 2009 Follicular development in pregnant cows after the administration of equine chorionic gonadotropin (eCG): A new insight. *Immunopharmacol.Immunotoxicol.*

- Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, Persons DL, Smith PG & Terranova PF 2000 Development of a syngeneic mouse model for events related to ovarian cancer. *Carcinogenesis* **21** 585-591.

- Rodriguez-Burford C, Barnes MN, Berry W, Partridge EE & Grizzle WE 2001 Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. *Gynecol.Oncol.* **81** 373-379.

- Rodriguez GC, Walmer DK, Cline M, Krigman H, Lessey BA, Whitaker RS, Dodge R & Hughes CL 1998 Effect of progestin on the ovarian epithelium of macaques: cancer prevention through apoptosis? *J.Soc.Gynecol.Investig.* **5** 271-276.

- Roland IH, Yang WL, Yang DH, Daly MB, Ozols RF, Hamilton TC, Lynch HT, Godwin AK & Xu XX 2003 Loss of surface and cyst epithelial basement

membranes and preneoplastic morphologic changes in prophylactic oophorectomies. *Cancer* **98** 2607-2623.

- Rossing MA, Daling JR, Weiss NS, Moore DE & Self SG 1994 Ovarian tumors in a cohort of infertile women. *N.Engl.J.Med.* **331** 771-776.

- Rotter V, Abutbul H & Ben Ze'ev A 1983 P53 transformation-related protein accumulates in the nucleus of transformed fibroblasts in association with the chromatin and is found in the cytoplasm of non-transformed fibroblasts. *EMBO J.* **2** 1041-1047.

- Rowe AJ, Morris KD, Bicknell R & Fraser HM 2002 Angiogenesis in the corpus luteum of early pregnancy in the marmoset and the effects of vascular endothelial growth factor immunoneutralization on establishment of pregnancy. *Biol.Reprod.* **67** 1180-1188.

- Roy SK & Treacy BJ 1993 Isolation and long-term culture of human preantral follicles. *Fertil.Steril.* **59** 783-790.

- Rutgers JL & Scully RE 1988 Cysts (cystadenomas) and tumors of the rete ovarii. *Int.J.Gynecol.Pathol.* **7** 330-342.

- Ryan KE, Glister C, Lonergan P, Martin F, Knight PG & Evans AC 2008 Functional significance of the signal transduction pathways Akt and Erk in ovarian follicles: in vitro and in vivo studies in cattle and sheep. *J.Ovarian.Res.* **1** 2.

- Ryan PL, Valentine AF & Bagnell CA 1996 Expression of epithelial cadherin in the developing and adult pig ovary. *Biol.Reprod.* **55** 1091-1097.

- Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT & Hamilton TC 1996 Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. *J.Natl.Cancer Inst.* **88** 1810-1820.

- Savio JD, Thatcher WW, Badinga L, de la Sota RL & Wolfenson D 1993 Regulation of dominant follicle turnover during the oestrous cycle in cows. *J.Reprod.Fertil.* **97** 197-203.

- Schildkraut JM, Bastos E & Berchuck A 1997 Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer. *J.Natl.Cancer Inst.* **89** 932-938.

- Schneider HP & Birkhauser M 1995 Does HRT modify risk of gynecological cancers? *Int.J.Fertil.Menopausal Stud.* **40 Suppl 1** 40-53.

- Scholler N, Crawford M, Sato A, Drescher CW, O'Briant KC, Kiviat N, Anderson GL & Urban N 2006 Bead-based ELISA for validation of ovarian cancer early detection markers. *Clin.Cancer Res.* **12** 2117-2124.

- Scholzen T & Gerdes J 2000 The Ki-67 protein: from the known and the unknown. *J.Cell Physiol* **182** 311-322.

- Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF, III & Amsterdam A 2001 The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. *J.Biol.Chem.* **276** 13957-13964.

- Shoham Z 1994 Epidemiology, etiology, and fertility drugs in ovarian epithelial carcinoma: where are we today? *Fertil.Steril.* **62** 433-448.

- Silva-Buttkus P, Jayasooriya GS, Mora JM, Mobberley M, Ryder TA, Baithun M, Stark J, Franks S & Hardy K 2008 Effect of cell shape and packing density on granulosa cell proliferation and formation of multiple layers during early follicle development in the ovary. *J.Cell Sci.* **121** 3890-3900.

- Simpson BJ, Langdon SP, Rabiasz GJ, Macleod KG, Hirst GL, Bartlett JM, Crew AJ, Hawkins RA, Macineira-Perez PP, Smyth JF & Miller WR 1998 Estrogen regulation of transforming growth factor-alpha in ovarian cancer. *J.Steroid Biochem.Mol.Biol.* **64** 137-145.

- Siskind V, Green A, Bain C & Purdie D 2000 Beyond ovulation: oral contraceptives and epithelial ovarian cancer. *Epidemiology* **11** 106-110.

- Sivachelvan MN, Ghali Ali M & Chibuzo GA 1996 Foetal age estimation in sheep and goats. *Small Ruminant Research* **19** 69-76.

- Slot KA, Boer-Brouwer M, Voorendt M, Sie-Go DM, Ghahremani M, Dorrington JH & Teerds KJ 2006 Irregularly shaped inclusion cysts display increased expression of Ki67, Fas, Fas ligand, and procaspase-3 but relatively little active caspase-3. *Int.J.Gynecol.Cancer* **16** 231-239.

- Smith VG, Edgerton LA, Hafs HD & Convey EM 1973 Bovine serum estrogens, progestins and glucocorticoids during late pregnancy parturition and early lactation. *J.Anim Sci.* **36** 391-396.

- Sowter HM & Ashworth A 2005 BRCA1 and BRCA2 as ovarian cancer susceptibility genes. *Carcinogenesis* **26** 1651-1656.

- Spritzer PM, Morsch DM & Wiltgen D 2005 [Polycystic ovary syndrome associated neoplasms]. *Arq Bras.Endocrinol.Metabol.* **49** 805-810.

- Stanislaus D, Janovick JA, Brothers S & Conn PM 1997 Regulation of G(q/11)alpha by the gonadotropin-releasing hormone receptor. *Mol.Endocrinol.* **11** 738-746.

- Steinetz BG, Randolph C & Mahoney CJ 1995 Patterns of relaxin and steroids in the reproductive cycle of the common marmoset (Callithrix jacchus): effects of prostaglandin F2 alpha on relaxin and progesterone secretion during pregnancy. *Biol.Reprod.* **53** 834-839.

- Stouffer RL, Martinez-Chequer JC, Molskness TA, Xu F & Hazzard TM 2001 Regulation and action of angiogenic factors in the primate ovary. *Arch.Med.Res.* **32** 567-575.

- Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM & Eppig JJ 2008 Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development* **135** 111-121.

- Syed V & Ho SM 2003 Progesterone-induced apoptosis in immortalized normal and malignant human ovarian surface epithelial cells involves enhanced expression of FasL. *Oncogene* **22** 6883-6890.

- Syed V, Ulinski G, Mok SC, Yiu GK & Ho SM 2001 Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. *Cancer Res.* **61** 6768-6776.

- Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R, Dombkowski D, Preffer F, Maclaughlin DT & Donahoe PK 2006 Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. *Proc.Natl.Acad.Sci.U.S.A* **103** 11154-11159.

- Szotek PP, Chang HL, Brennand K, Fujino A, Pieretti-Vanmarcke R, Lo CC, Dombkowski D, Preffer F, Cohen KS, Teixeira J & Donahoe PK 2008 Normal ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics. *Proc.Natl.Acad.Sci.U.S.A* **105** 12469-12473.

- Tan OL & Fleming JS 2004 Proliferating cell nuclear antigen immunoreactivity in the ovarian surface epithelium of mice of varying ages and total lifetime ovulation number following ovulation. *Biol.Reprod.* **71** 1501-1507.

- Tannenbaum PL, Schultz-Darken NJ, Woller MJ & Abbott DH 2007 Gonadotrophin-releasing hormone (GnRH) release in marmosets II: pulsatile release of GnRH and pituitary gonadotrophin in adult females. *J.Neuroendocrinol.* **19** 354-363.

- Taya K & Greenwald GS 1981a Effect of hypophysectomy on day 12 of pregnancy on ovarian steroidogenesis in the rat. *Biol.Reprod.* **25** 692-698.

- Taya K & Greenwald GS 1981b In vivo and in vitro ovarian steroidogenesis in the pregnant rat. *Biol.Reprod.* **25** 683-691.

- Taylor PD, Hillier SG & Fraser HM 2004 Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. *J.Endocrinol.* **183** 1-17.

- Telfer EE, Gosden RG, Byskov AG, Spears N, Albertini D, Andersen CY, Anderson R, Braw-Tal R, Clarke H, Gougeon A, McLaughlin E, McLaren A, McNatty K, Schatten G, Silber S & Tsafriri A 2005 On regenerating the ovary and generating controversy. *Cell* **122** 821-822.

- Telfer EE & McLaughlin M 2007 Natural history of the mammalian oocyte. *Reprod.Biomed.Online.* **15** 288-295.

- Telfer EE, McLaughlin M, Ding C & Thong KJ 2008 A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum.Reprod.* **23** 1151-1158.

- Tiedemann D 2000 Oncology today: ovarian cancer. *RN.* **63** 36-41.

- Tomanek M & Chronowska E 2006 Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the pig ovary. *Folia Histochem.Cytobiol.* **44** 269-274.

- Tonetta SA & diZerega GS 1989 Intragonadal regulation of follicular maturation. *Endocr.Rev.* **10** 205-229.

- Toosi BM, Seekallu SV, Barrett DM, Davies KL, Duggavathi R, Bagu ET & Rawlings NC 2010 Characteristics of peaks in serum concentrations of follicle-stimulating hormone and estradiol, and follicular wave dynamics during the interovulatory interval in cyclic ewes. *Theriogenology* **73** 1192-1201.

- Travali S, Ku DH, Rizzo MG, Ottavio L, Baserga R & Calabretta B 1989 Structure of the human gene for the proliferating cell nuclear antigen. *J.Biol.Chem.* **264** 7466-7472.

- Tung KH, Wilkens LR, Wu AH, McDuffie K, Nomura AM, Kolonel LN, Terada KY & Goodman MT 2005 Effect of anovulation factors on pre- and postmenopausal ovarian cancer risk: revisiting the incessant ovulation hypothesis. *Am.J.Epidemiol.* **161** 321-329.

- Turzillo AM & Fortune JE 1993 Effects of suppressing plasma FSH on ovarian follicular dominance in cattle. *J.Reprod.Fertil.* **98** 113-119.

- Tutt A & Ashworth A 2002 The relationship between the roles of BRCA genes in DNA repair and cancer predisposition. *Trends Mol.Med.* **8** 571-576.

- Umar A, Buermeyer AB, Simon JA, Thomas DC, Clark AB, Liskay RM & Kunkel TA 1996 Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. *Cell* **87** 65-73.

- van der Linden PJ, de Goeij AF, Dunselman GA, Arends JW & Evers JL 1994 P-cadherin expression in human endometrium and endometriosis. *Gynecol.Obstet.Invest* **38** 183-185.

- van Tol HT & Bevers MM 2001 Partial characterization of the factor in theca-cell conditioned medium that inhibits the progression of FSH-induced meiosis of bovine oocytes surrounded by cumulus cells connected to the membrana granulosa. *Mol.Reprod.Dev.* **60** 418-424.

- van den BW, Coryn M, Simoens P & Lauwers H 2002 Cell-specific distribution of oestrogen receptor-alpha in the bovine ovary. *Reprod.Domest.Anim* **37** 291-293.

- Vanderhyden BC, Shaw TJ & Ethier JF 2003 Animal models of ovarian cancer. *Reprod.Biol.Endocrinol.* **1** 67.

- Wassarman PM 1988 Zona pellucida glycoproteins. *Annu.Rev.Biochem.* **57** 415-442.
- Whittemore AS, Harris R & Itnyre J 1992a Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. IV. The pathogenesis of epithelial ovarian cancer. Collaborative Ovarian Cancer Group. *Am.J.Epidemiol.* **136** 1212-1220.

- Whittemore AS, Harris R & Itnyre J 1992b Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. *Am.J.Epidemiol.* **136** 1184-1203.

- Wilcox CB, Feddes GO, Willett-Brozick JE, Hsu LC, DeLoia JA & Baysal BE 2007 Coordinate up-regulation of TMEM97 and cholesterol biosynthesis genes in normal ovarian surface epithelial cells treated with progesterone: implications for pathogenesis of ovarian cancer. *BMC.Cancer* **7** 223.

- Wimalasena J, Meehan D, Dostal R, Foster JS, Cameron M & Smith M 1993 Growth factors interact with estradiol and gonadotropins in the regulation of ovarian cancer cell growth and growth factor receptors. *Oncol.Res.* **5** 325-337.

- Wong AS & Auersperg N 2003 Ovarian surface epithelium: family history and early events in ovarian cancer. *Reprod.Biol.Endocrinol.* **1** 70.

- Wong AS & Leung PC 2007 Role of endocrine and growth factors on the ovarian surface epithelium. *J.Obstet.Gynaecol.Res.* **33** 3-16.

- Wong AS, Roskelley CD, Pelech S, Miller D, Leung PC & Auersperg N 2004 Progressive changes in Met-dependent signaling in a human ovarian surface epithelial model of malignant transformation. *Exp.Cell Res.* **299** 248-256.

- Wong AS, Maines-Bandiera SL, Rosen B, Wheelock MJ, Johnson KR, Leung PC, Roskelley CD & Auersperg N 1999 Constitutive and conditional cadherin expression in cultured human ovarian surface epithelium: influence of family history of ovarian cancer. *Int.J.Cancer* **81** 180-188.

- Wright JW, Pejovic T, Fanton J & Stouffer RL 2008 Induction of proliferation in the primate ovarian surface epithelium in vivo. *Hum.Reprod.* **23** 129-138.

- Wright JW, Stouffer RL & Rodland KD 2003 Estrogen inhibits cell cycle progression and retinoblastoma phosphorylation in rhesus ovarian surface epithelial cell culture. *Mol.Cell Endocrinol.* **208** 1-10.

- Wright JW, Stouffer RL & Rodland KD 2005 High-dose estrogen and clinical selective estrogen receptor modulators induce growth arrest, p21, and p53 in primate ovarian surface epithelial cells. *J.Clin.Endocrinol.Metab* **90** 3688-3695.

- Wu HM, Wang HS, Huang HY, Soong YK, MacCalman CD & Leung PC 2009 GnRH signaling in intrauterine tissues. *Reproduction.* **137** 769-777.

- Wulff C, Wiegand SJ, Saunders PT, Scobie GA & Fraser HM 2001 Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). *Endocrinology* **142** 3244-3254.

- Yang DH, Smith ER, Cohen C, Wu H, Patriotis C, Godwin AK, Hamilton TC & Xu XX 2002 Molecular events associated with dysplastic morphologic transformation and initiation of ovarian tumorigenicity. *Cancer* **94** 2380-2392.

- Yen SSC 1994 Endocrinology of pregnancy. In Creasy, R.K. and Resnik,R. (eds), *Maternal–fetal Medicine: Principals and Practice.* W.B. Saunders, *Philadelphia*. 382–412.

- Zeleznik AJ, Saxena D & Little-Ihrig L 2003 Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. *Endocrinology* **144** 3985-3994.